이학박사학위논문

Identification of SGK1 as a novel positive regulator of YAP and TAZ

SGK1 에 의한 YAP 과 TAZ 의 활성조절 연구

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ABSTRACT

Identification of SGK1 as a novel positive regulator of YAP and TAZ

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In recent years, an emerging role of YAP and TAZ in the increasingly sophisticated cancer biology field demands a more articulate understanding of protein networks surrounding YAP and TAZ. This also means elucidation of detailed regulatory mechanisms relevant to YAP/TAZ within the pre-established signaling pathway, is crucial to better understand the extent of YAP and TAZ's activity during cancer development.

With this in mind, I worked on candidate regulatory partners of YAP and TAZ. SGK1 is an oncoprotein of the PI3-K signaling pathway with recent therapeutic implication in AKT inhibitor-resistant cancers. I confirmed that SGK1 is a downstream target of YAP with rapid responsiveness to YAP activation. Moreover, SGK1 transcription was

controlled by a critical YAP-interacting transcription factor, TEAD. In addition, I found that YAP directly binds to the distal enhancer of SGK1 to promote transcription. On the other hand, I discovered that SGK1 controls canonical YAP/TAZ targets, CTGF and CYR61 via its kinase domain and PPxY motif. Furthermore, SGK1 regulates YAP and TAZ protein levels to promote CTGF and CYR61 expression. Lastly, I found that SGK1 stabilizes TAZ via GSK3β phosphorylation.

Alternatively, I discovered that TAZ is regulated by its paralog, YAP, in a negative way. I found that this regulation is rather unidirectional and conserved among numerous cell lines. TAZ regulation by YAP was controlled at the translational level, but not at the transcriptional or post-translational level. Finally, I found this regulatory mechanism was independent of the Hippo signaling pathway.

In conclusion, I discovered two regulatory mechanisms controlling TAZ in both positive and negative manners.

Keywords : SGK1, YAP, TAZ, CTGF, CYR61, Hippo pathway

***Student Number* : 2008-22750**

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ABBREVIATIONS

AKT	Ak Mouse Strain Thymoma
ATP	Adenosine triphosphate
BIO	6-bromoindirubin-3'-oxime
ChIP	Chromatin Immunoprecipitation
CHX	Cycloheximide
CK1	Casein kinase 1
CTGF	Connective growth factor
CYR61	Cysteine-rich angiogenic inducer 61
dAkt	The *Drosophila* AKT
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ER^{t2}	Estrogen receptor variant
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEO	Gene expression omnibus
GSK3	Glycogen synthase kinase 3
H3K27	Histone 3 Lysine 27
IRES	Internal ribosome entry site
KO	Knock-out
K127R	Lysine 127 Arginine
LATS1	Large tumor suppressor kinase 1

LiCl	Lithium chloride
M-MLV	Moloney Murine Leukemia virus
MST1	Mammalian sterile 20–like kinase-1
mTOR	Mammalian target of rapamycin
NF2	Neurofibromatosis 2
qPCR	quantitative polymerase chain reaction
PDK	Phosphoinositide-dependent protein kinase
PDZ	PSD95, Dlg1, Zo-1
PH	Pleckstrin homology
PI3-K	Phosphoinositide-3-kinase
PIP3	phosphatidylinositol 3,4,5 trisphosphate
PPxY	Proline-Proline-x-Tyrosine
RIPA	Radioimmunoprecipitation assay
RNAi	RNA interference
RPE1	Retinal pigment epithelial cell line 1
SDS	Sodium dodecyl sulfate
S.E.M.	Standard error of the mean
SGK1	Serum and glucocorticoid-inducible kinase 1
sgRNA	single guide RNA
shRNA	small hairpin RNA
SH3	SRC homology 3
siRNA	small interfering RNA
S94A	Serine 94 Alanine
S1079	Serine 1079
TAZ	Transcription coactivator with PDZ-binding motif

TEAD	TEA domain family member transcription factor
TRC	The RNAi Consortium
TSS	Transcription start site
WT	Wildtype
WW	WWP repeating motif
YAP	Yes-associated protein
Yki	Yorkie
Y298A	Tyrosine 298 Alanine
5SA	5 Serine to Alanine

INTRODUCTION

The Hippo pathway is a universally conserved tumor suppressor pathway conserved from flies to human. Originally identified in *Drosophila, Hippo* of the Hippo pathway is a tumor-suppressive upstream kinase of *Warts*, which is the other tumor-suppressive upstream kinase of *Yorkie*, the transcriptional regulator. Overexpression of *Yorkie* in *Drosophila* wing disc resulted in uncontrolled overgrowth, while promoted cell proliferation and anti-apoptosis in eye disc, suggesting an oncogenic role of *Yorkie*. (Huang et al., 2005) The mammalian homologs of *Yorkie*, YAP and TAZ are the effector molecules of the Hippo pathway that controls organ size, tissue regeneration upon injury and cancer development. (Harvey et al., 2013; Johnson and Halder, 2014). Due to their role in the initiation and the progression of cancer in many types of organs, YAP and TAZ are implicated in cancer therapy (Janse van Rensburg et al., 2016; Zanconato et al., 2016a).

Until recent, YAP and TAZ are shown to be solely regulated by their upstream kinases, LATS1 and LATS2, reflecting the canonical Hippo dependent regulation on YAP and TAZ. For example, Hippo signaling is activated by basolateral polarity organizing proteins such as Scribble in high cell density context and inactivated by GPCR (G-Protein coupled Receptor) upon a serum stimulation (Condenonsi et al., 2011; Yu et al., 2012). However, intriguing works by Piccolo and colleagues showed the Hippo pathway independent regulation of YAP and TAZ by

transducers of mechanical signals (Dupont et al., 2011, Aragona et al., 2013). This opened up the discussion on the regulation of YAP and TAZ in the Hippo pathway independent mechanism.

Although YAP and TAZ are highly expressed in cancers of many organs, Hippo upstream regulators are rarely mutated in human cancers, while mutations in NF2 or LATS1 or LATS2 were present in only certain tumor histotypes (Harvey et al., 2013; Zanconato et al., 2016b). This suggested additional mode of YAP/TAZ regulation may be involved in YAP and TAZ driven cancers. Not surprisingly, recent works uncovered cooperative relations of the Hippo pathway with Wnt, TGFβ, Notch, mevalonate, and EGF signaling (Hansen et al., 2015; Zhou et al., 2016).

In following studies, I worked on uncovering hidden branches of regulatory mechanisms of YAP and TAZ. Surprisingly, I discovered a dual role of SGK1 as a downstream target gene of YAP, and positive regulator of YAP and TAZ. Moreover, I also worked on regulatory relations of YAP and TAZ. I found that YAP regulates TAZ in unidirectional mode, while this regulation is controlled at the translational level via a LATS1/2 independent mechanism.

CHAPTER I

Identification of SGK1 as a novel positive regulator of YAP/TAZ

Abstract

The importance of YAP/TAZ in the field of tissue regeneration and cancer necessitates the discovery of their new regulatory partners. Initially identified as a homolog of AKT in PI3K signaling, SGK1 acts as a serine/threonine protein kinase in multiple oncogenic pathways. However, the possible link between SGK1 and YAP/TAZ oncoproteins remains unexplored. Here, I investigated whether SGK1 serves as a positive regulator of YAP/TAZ, utilizing a standard molecular cell biology approach. Indeed, SGK1 levels are positively correlated with levels of YAP/TAZ target genes, CTGF and CYR61 in MCF-10A cells. Mechanistically, SGK1 elevates YAP/TAZ protein levels to enhance their activity. As I investigated further, I found that SGK1 stabilizes TAZ via SGK1-GSK3ß-TAZ inhibitory circuit. I also accumulated evidence that YAP controls SGK1 expression, retrospectively.

Introduction

YAP (Yes-Associated Protein) is an oncogenic transcriptional coactivator. YAP was first identified as a protein that contains a protein interaction motif called the WW domain (Sudol et al., 1995). Studies thereafter revealed that YAP is a transcriptional coactivator which binds transcription factors for enhancement of transcriptional output (Yagi et al., 1999). Therefore, the physiological functions of YAP depend on which transcription factor it binds to. Suggesting its versatility, YAP not only has a WW domain, but also has TEAD-binding, PDZ-binding, SH3 domain-binding motifs (Wang et al., 2009).

As a proto-oncogene found in the 11q22 amplicon that is frequently observed in multiple human cancers, YAP promotes invasion and proliferation, inhibits apoptosis and is sufficient for to cause cellular transformation (Overholtzer et al., 2006). Likewise, TAZ (Transcriptional coactivator with PDZ binding motif), a paralog of YAP, is known to have similar functions as YAP in terms of cell proliferation, anti-apoptosis, and cell-transforming property (Chan et al., 2008). Both YAP and TAZ are mammalian homologs of Yorkie, the downstream effector protein of newly emerging tumorsuppressor pathway called the Hippo pathway (Zhao et al., 2010). The Hippo pathway has been shown to be conserved in many metazoan species ranging from *Drosophila* to mammals. Therefore, understanding the function and regulatory partners of YAP and TAZ may provide important insights to

an evolutionarily conserved regulatory mechanism of cell proliferation and cell survival.

SGK1 (Serum- and glucocorticoid inducible kinase 1) belongs to the AGC family of serine/threonine protein kinases, which also includes AKT1 (protein kinase B) and protein kinase C as well as SGK1's close paralogs, SGK2 and SGK3. As one can tell from its name, SGK1 level is regulated by serum and glucocorticoids, in addition to various stimuli (Waldegger et al., 1997, Leong et al., 2003). SGK1's enzymatic activity is controlled by a phosphorylation cascade initiated by PI3K (Phosphoinositide-3 kinase) activation, and executed by PDKs (Phosphoinositide-dependent protein kinases).

Recently, *Drosophila* genetics study suggested that Yorkie, the *Drosophila* homolog of YAP and TAZ, positively regulates *dAkt* (Ye et al., 2012). *dAkt* is an oncogenic kinase induced by insulin and is a Drosophila homolog of a mammalian protein kinases, AKT1 and SGK1, with which it shares high sequence similarity. Intriguingly, both SGK1 and AKT1 phosphorylate and control the activity of many of the same target proteins (Wu et al., 2004, Aoyama et al., 2005). Both AKT1 and SGK1 are known to promote cell proliferation, and cell survival (Brunet et al. 2001, Bruhn et al. 2010). Even though AKT1 and SGK1 have these similarities, they differ in their functional domain. For example, AKT1 contains a PH (Pleckstrin Homology) domain that is required for the PI3K-dependent plasma membrane localization, while SGK1 does not have a PH domain which explains why SGK1 may remain active in the absence of PIP3 (Castel et al., 2016). Therefore, distinguishing

features of SGK1 and AKT1 suggests an additional layer of complexity we researchers should be aware of when considering therapeutic options in AKT or SGK1-activated cancers.

The similarity between aforementioned AKT1 or SGK1 activation and the phenotype observed by YAP overexpression, with the above fly genetics data, suggests that AKT1 or SGK1 could be a compelling target of YAP induced tumorigenesis in mammals. However, evidence of whether SGK1 could be regulated analogously by YAP as *dAkt* by *Yorkie* in *Drosophila* remains lacking.

Next, there are similarities between YAP and SGK1. First, SGK1 is involved in multiple oncogenic signaling cascades, including the mTOR and PI3K signaling pathway (Park et al., 1999, Hong et al., 2008), while recent studies suggested a crosstalk between YAP-Hippo pathway and mTOR/PI3K pathway (Tumaneng et al., 2012, Mo et al., 2015). In addition, while SGK1 was initially identified as protein kinase upregulated by serum (Webster et al., 1993), YAP and TAZ were recently shown to be activated upon stimulation with serum (Yu et al., 2012). However, the possible link between SGK1 and YAP/TAZ onco-proteins remains unexplored. It would be interesting to find out whether a regulatory relationship exists between SGK1 and the YAP/TAZ-Hippo pathway.

In this study, I found that SGK1 is a downstream target gene of YAP. The TEAD binding motif of YAP was responsible for controlling SGK1 transcriptional expression. In addition, YAP directly binds to SGK1 enhancer region which contains the TEAD consensus motif,

CATTCC. Physiologically, SGK1 supports YAP function in anchorage independent growth. On the other hand, SGK1 positively regulates YAP/TAZ. SGK1 enhances YAP/TAZ target genes, CTGF and CYR61. More interestingly, SGK1 up-regulates YAP and TAZ levels. SGK1 stabilizes TAZ protein by inhibition of SGK1 substrate, GSK3β, which also happens to be a negative regulator of TAZ. These data suggests SGK1 is a novel positive regulator of YAP/TAZ.

Materials and Methods

Cell culture and Drug treatments

MCF-10A was cultured in DMEM/F12 media supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml and insulin as completed medium. 293T and HEK293 were cultured in DMEM supplemented with 10% FBS. All of these media contained antibiotics (penicillin/streptomycin). In case of SGK1 knockdown sets, cells were split to reach confluence at the time of harvest, while for SGK1 overexpression experiments, cells were split to stay sub-confluence when YAP/TAZ are active.

For drug treatments, the following compounds and concentrations were used: MG132 (50 μM, 6 h), GSK650394 (1 μM, 2.5 μM, 5 μM, 24 h), Lithium Chloride (25 mM, 24 h), BIO (8 μM, 24 h)

Lentiviral SGK1 Overexpression plasmids

pCMV-SGK1 plasmid was a kind gift from Dr. Eui-Ju Choi. WT SGK1 was cloned into *pLVX IRES puro* (purchased from Addgene) by BamH1. Kinase-dead SGK1, K127R SGK1 and WW domain binding defective SGK1, Y298A SGK1 were generated by nested PCR technique.

Primers used for SGK1 mutation are following: K127R SGK1 Forward (5-ATGCAGTCAAGGTTTTACAGA-3), K127R SGK1 Reverse (5-TCT GTA AAA CCT TGA CTG CAT-3), Y298A SGK1 Forward (5-GCCTGCCGCCTTTTGCTAGCCGAAACACAG-3) and Y298A SGK1

Reverse (5-CTGTGTTTCGGCTAGCAAAAGGCGGCAGGC-3). To generate lentivirus, *pLVX IRES puro SGK1*, *pCMV-dR8.2 dvpr* and *pCMV-VSVG* were transfected into 293T cells with polyethyleneimine. After 6-hour incubation, initial medium was changed to fresh medium, and incubated for 48 h. On the harvest day, lentiviral supernatant was collected to be infected into target cell line with polybrene (9 μg/ml). After a day, viral supernatant was replaced to normal medium.

shRNA infection and shRNA sequences

To produce retroviruses, *pSuper retro* (puro), *pCMV-Gag-Pol* and *pCMV-VSVG* were transfected into 293T cells with polyethyleneimine. Two days later, the media were harvested and centrifuged for 20 min at 3,000 g. For retrovirus infection, the harvested media were added into the same volume of normal culture media with polybrene (9 μg/ml), cultured for 24 h and replaced with a normal culture medium. Two days after the infection, puromycin(0.5 μg/ml) was added for selection. *pSuper retro shGFP and pSuper retro shSGK1 A* were gifted from Dr. Eui-ju Choi (Kim et al., 2007). shSGK1 is *shSGK1 A*, otherwise indicated.

shRNA sequences used in this study are *shGFP* (5-GGCUACGUCCAGGAGCGCACC-3), *shSGK1 A* (5-GTCCTTCTCAGCAAATCAACC-3) and *shSGK1 B* (5-CGGAATGTTCTGTTGAAGAA-3). *shSGK1 B* sequence was obtained from Sommer et al. (Sommer et al., 2013) which has TRC number of TRCN0000194957.

siRNA transfection and siRNA sequnces

Lipofectamine RNAiMAX Reagent (Life Technologies) was used for transfection of siRNA. siRNA was prepared and transfected as described by the manufacturer. RNA oligonucleotides were synthesized by Samchully Pharmaceutical Co. siRNA sequences used in this study are siControl(GL2) (5-CGTACGCGGAATACTTCGA-3), siYAP (5-GACAUCUUCUGGUCAGAGA-3), and siTAZ (5-ACGUUG ACUUAGGAACUUU-3). siYAP and siTAZ sequences were obtained from Kim et al. (Kim et al., 2015b).

Retrovirus generation for YAP 5SA Overexpression

pMSCV hygro, pMSCV hygro flag 5SA YAP and *pMSCV hygro flag 5SA S94A YAP* plasmid were used for control or 5SA or 5SA S94A YAP overexpression experiments. Plasmids were transfected into 293T cells to produce retroviruses used for target cell infection.

Western blot

Cells were harvested and lysed with RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) containing protease inhibitors and phosphatase inhibitors (1 μg/ml Pepstatin A, 1 μg/ml Leupeptin, 1 mM Phenylmethylsulfonyl Fluoride, 1 mM Sodium Orthovanadate, 5 mM Sodium Fluoride). Western blot analyses were performed using a standard protocol.

Quantitative PCR

RNA preparation and cDNA synthesis were done as described by the manufacturer using RiboEx (GeneAll) and M-MLV reverse transcriptase (Enzynomics). Quantitative polymerase chain reaction (qPCR) was performed using a SYBR green premix reagent (TOPreal qPCR 2X PreMIX; Enzynomics) and Bio-Rad CFX Connect instrument. Results were analyzed using Microsoft Excel.

Primers used for quantitative PCR are following: GAPDH Forward (5-CTTCGCTCTCTGCTCCTCCT-3), GADPH Reverse (5-GTTAAAAGCAGCCCTGGTGA-3), SGK1 Forward (5-CATATTATGTCGGAGCGGAATGT-3), SGK1 Reverse (5-TGTCAGCAGTCTGGAAAGAGA-3), CTGF Forward (5-CCAATGACAACGCCTCCTG-3), CTGF Reverse (5-TGGTGCAGCCAGAAAGCTC-3), CYR61 Forward (5-GGTCAAAGTTACCGGGCAGT-3), CYR61 Reverse (5-GGAGGCATCGAATCCCAGC-3), YAP Foward (5-GAACCAGAGAATCAGTCAGA-3), YAP Reverse (5-GGATTGATATTCCGCATTGC-3), TAZ Forward (5-GTCCTACGACGTGACCGAC-3), and TAZ Reverse (5-CACGAGATTTGGCTGGGATAC-3).

Antibodies

Antibodies used for western blot analysis include: YAP (raised against the C-terminal human YAP antigen by Kim et al. (Kim et al. 2015)), SGK1 (D27C11) (Cell Signaling Tech.), CTGF (Santa Cruz Biotech.),

CYR61 (Santa Cruz Biotech.), ß-actin (Sigma), p-YAP (Ser127) (Cell Signaling Tech.), TAZ (Cell Signaling Tech.), p-GSK3α/β (Cell Signaling Tech.), and Ubiquitin (Santa Cruz Biotech.), LATS2 (Cell Signaling Tech.), and GSK3α/β (Cell Signaling Tech).

Luciferase assay

Luciferase constructs and each indicated DNA construct were co-transfected with a Renilla luciferase construct, used as a control for transfection efficiency. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's guide. Luciferase signal intensities were calculated relative to those of Renilla luciferase using Microsoft Excel.

***in vivo* ubiquitination assay**

In vivo ubiquitination assay was performed in high stringency IP buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate) with 0.1% SDS to dissociate TAZ interacting proteins from TAZ during ubiquitination reaction. For immunoprecipitation of TAZ, anti-TAZ antibody (Santa Cruz Biotech.) was used instead of anti-TAZ antibody (Cell Signaling Tech.) due to stronger binding affinity.

Chromatin Immunoprecipitation

Two confluent 100-mm culture dishes (2~3x10^7 cells) were washed with PBS and DNA of these cells were cross-linked with 1%

formaldehyde for 15 minutes. Cells were then processed for ChIP assay as previous described by Kim et al. (Kim et al. 2015c) using anti-Flag antibody (Sigma F1804) (5 μg) and Protein A/G agarose (GenDEPOT) (20 μl). Relative enrichment in SGK1 regulatory sequences and CTGF promoters were measured as a ratio to enrichment in the gene desert in chromosome 12.

The following primers were used to detect the indicated genomic regions:

SGK1 7807 ChIP Forward (5-CTGCACCAGGGAAAATGC-3), SGK1 7807 ChIP Reverse (5-GATTTGGCTTTTCTTTGACCA-3), CTGF ChIP Forward (5-CAATCCGGTGTGAGTTGATG-3), CTGF ChIP Reverse (5-GCCAATGAGCTGAATGGAGT-3), Gene Desert 12 ChIP Forward (5-TTCCAAGCGGTAAAGCTTC-3), and Gene Desert 12 ChIP Reverse (5-TCCCAAAGTGCTGGGATTAC-3). Sequences for Gene desert 12 were obtained from Kim et al. (Kim et al., 2015c). 5SA YAP was used for optimal binding to chromatin.

Gene Expression Omnibus (GEO) microarray analysis

Microarray data for YAP hyperactive overexpression was downloaded from NCBI GEO. Microarray data was normalized and analyzed with Morpheus (Broad Institute). The Microarray used for this study is GSE60579 (Kim et al. 2015).

ChIP sequencing (GEO and unpublished) analysis

Raw and processed ChIP sequencing data for overexpressed YAP

enrichment in the genome was downloaded from NCBI GEO. ChIP sequencing data were uploaded onto UCSC genome browser (http://Genome.ucsc.edu/). The ChIP sequencings used for this study were GSE61852 (Stein et al. 2015) and unpublished ChIP sequencing data by Dr. Dae-Sik Lim (KAIST).

Soft agar assay

Cell culture grade agar (Sigma) was dissolved in sterile water using a microwave. Bottom agar in media (0.5% agar) was plated in the wells of a 6-well plate. Trypsinized cells ($2x10^4$ cells per well) were then suspended in pre-warmed top agar in media (0.4% agar), then plated atop the bottom agar. Medium (2 ml per well) was changed to fresh medium every day, and incubated for 18 days. Colonies were stained with crystal violet. Images were acquired for one random region per well. The number and size of colonies were analyzed using the ImageJ program.

Statistical analysis

Two-tailed *t*-tests were used for statistical analyses. All analyses were performed using Prism4 (GraphPad Software). Error bars indicate S.E.M. unless otherwise specified. A *p*-value less than 0.05 indicates statistical significance.

RESULTS

SGK1 is a *bona fide* downstream target of YAP in MCF-10A cells.

Initially, I started my investigation searching for a putative relationship between SGK1 and YAP, by noticing that SGK1 was a candidate YAP target gene. Kim et al. previously performed a microarray analysis of MCF-10A-overexpressing Estrogen Receptor Domain (ERt2) fused-YAP 2SA (YAP with intermediate level of hyper-activation), so that YAP target genes would readily respond upon administration of the Estrogen analog, 4-Hydrooxytamoxifen (Kim et al., 2015c). From analyzing SGK1 gene expression profile at early time points of 4-Hydrooxytamoxifen treatment, I noticed that SGK1 very readily responded to the estrogen analog, as early as 2 h post-treatment. SGK1 was identified as an immediate early gene to serum and glucocorticoid (Webster et al., 1993). Thus, I found that SGK1 as one of genes that was quickly and strongly upregulated by YAP (Figure1.1A). Moreover, other groups' microarray data including that of Kim et al., consistently suggested SGK1 as a candidate YAP signature gene, by showing increase of SGK1 expression by approximately 3- to 5-fold in response to overexpression of various forms of YAP in mouse livers, mouse fibroblasts, and human mammary epithelial cells (Figure 1.1B) (Dong et al., 2007, Zhao et al., Kim et al., 2015c).

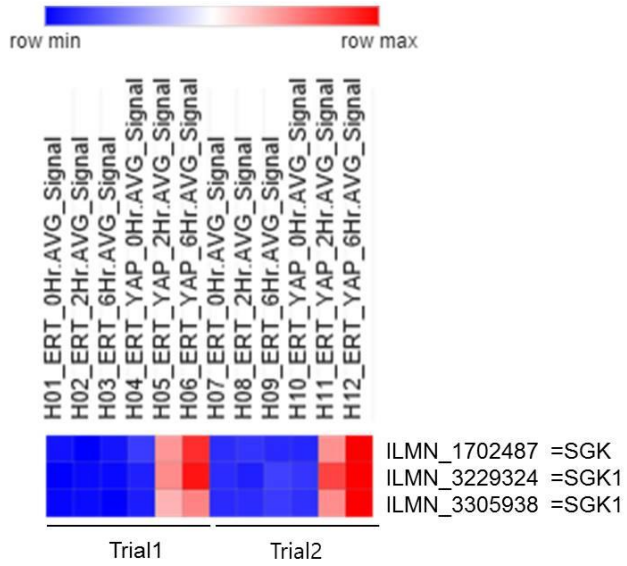
In order to confirm that SGK1 is a *bona fide* downstream target of YAP, I overexpressed various forms of YAP, each known to have

Figure 1-1. Numerous studies suggest SGK1 as a candidate downstream target of YAP.

(A) Heat-map analysis of SGK1 in MCF-10A ER^{t2}-YAP2SA microarray by Kim et al. (Kim et al., 2015). GSE60579 by Kim et al. was normalized and selected for a gene corresponding to SGK1.

(B) Three independent groups showing that SGK1 is increased upon overexpression of different forms of YAP.

A



GEO60579 Microarray analysis

B

Study	Form of YAP overexpression	Fold increase in SGK1 level
Dong et al., 2007	Doxycycline inducible WT YAP	5.744
Zhao et al., 2008	5SA YAP	3.13
Kim et al., 2015	Tamoxifen inducible 2SA YAP	3.86

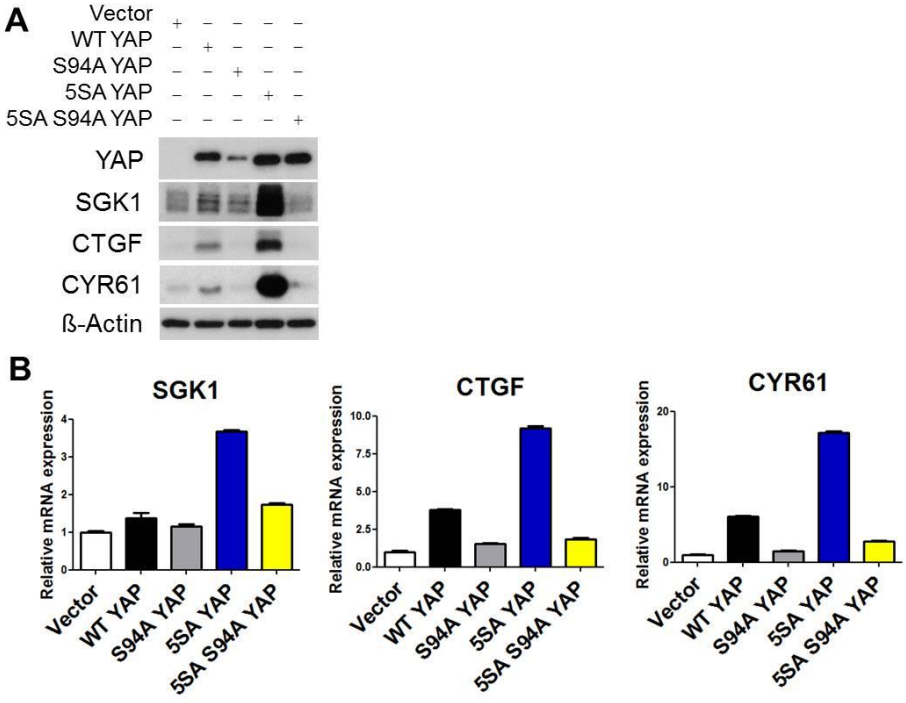
altered activities. I overexpressed WT YAP, 5SA YAP that is known thus far to be most hyperactive, S94A YAP that is TEAD transcription factor binding deficient (thus inactive due to inability to bind to DNA), and 5SA S94A YAP that is TEAD-binding deficient and thus no longer hyperactive (Zhao et al., 2008). Using Western blot and Quantitative real time PCR, I observed that SGK1 was somewhat significantly increased by WT YAP overexpression while 5SA YAP dramatically increased SGK1 in both protein and transcript levels, where a similar pattern could be observed in canonical YAP target genes, CTGF and CYR61 (Figure 1.2A-B). On the other hand, SGK1 upregulation was abolished to a nearly basal state level in both S94A YAP and S94A 5SA YAP (Figure 1.2A-B). These data point to two things: First, SGK1 expression by YAP is synchronized to other well-known YAP target genes, CTGF and CYR61. Second, SGK1 transcript and protein levels are tightly affected by YAP's ability to form the TEAD-YAP transcription factor-coactivator complex, which is known to be the most critical transcription factor affecting YAP activity (Zhao et al., 2008). Collectively, our data strongly support that SGK1 is a valid downstream target of YAP and this transcriptional control is TEAD-dependent.

To more closely examine the relationship between SGK1 and YAP relationship, I tried to figure out whether YAP amplifies SGK1 transcript directly or indirectly. Although evidence for immediate SGK1 response to YAP activation suggests that SGK1 is directly regulated by YAP, it was not conclusive. Thus, I searched for clues as to whether YAP is enriched in the SGK1 promoter region. Kim et al. (Kim et al.,

Figure 1-2. YAP hyperactivity drastically increases SGK1 in a TEAD dependent manner.

(A) Western blot analysis of SGK1 and YAP targets in stable cell lines that were generated by infecting either Control Vector or YAP packaged retroviruses (WT, S94A, 5SA, 5SA S94A) into MCF-10A and later selected with puromycin.

(B) Quantitative PCR analysis of SGK1 and YAP target genes in same sets as in (A). Each gene was normalized to GAPDH.


A
Vector
WT YAP
S94A YAP
5SA YAP
5SA S94A YAP
YAP
SGK1
CTGF
CYR61
ß-Actin
B
SGK1
CTGF
CYR61
Relative mRNA expression
Vector
WT YAP
S94A YAP
5SA YAP
5SA S94A YAP

2015c) recently performed YAP Chromatin Immunoprecipitation Sequencing in MCF-10A cells (Unpublished data), from which I decided to search for SGK1 chromatin region for YAP-binding peaks. Unfortunately, I could not detect a statistically significant YAP peak near the SGK1 transcription start site. However, I did see significant YAP peaks in SGK1 regulatory sequences in the chromatin. The regulatory sequences of a particular gene may consist of not only the promoter, but also enhancer region. I found there were three significant YAP peak intervals in the SGK1 regulatory sequence region marked by oranage lines (Figure 1.3). Among them, the YAP peak I named 7807, is approximately 120 kb away from the nearest SGK1 transcript variant, and approximately 250 kb away from the SGK1 TSS (Transcription start site). YAP peak 7807 showed the strongest peak intensity among the three YAP peaks (Figure 1.3).

There has been a few recent papers that suggested that YAP binds to enhancer regions of most target genes and activates them by chromatin looping (Zanconato et al., 2015, Galli et al., 2015, Stein et al., 2015). YAP peak 7807 region fits into this criterion as an enhancer region. Surprisingly, independent YAP ChIP sequencing data from Bauer group (Stein et al., 2015) showed the strongest YAP peak in the approximately the same region as YAP peak 7807 of SGK1 genomic region in three different cell lines they analyzed (Figure 1.4). The three cell lines they used were: IMR90 which is non-transformed human fibroblast, and the other two were SF268 and NCI-H2052, cancer cell lines of brain and lung, respectively (Stein et al., 2015). Therefore, a

Figure 1-3. YAP ChIP sequencing result indicates YAP is enriched in region "7807" of distal SGK1 regulatory sequences.

SGK1 regulatory sequences in line with YAP bound regions. YAP ChIP-sequencing data (raw and significant peaks) (Unpublished data provided by Dr. Dae-Sik Lim) were loaded into UCSC genome browser and strongest peak interval in SGK1 genomic region was assigned as "7807."

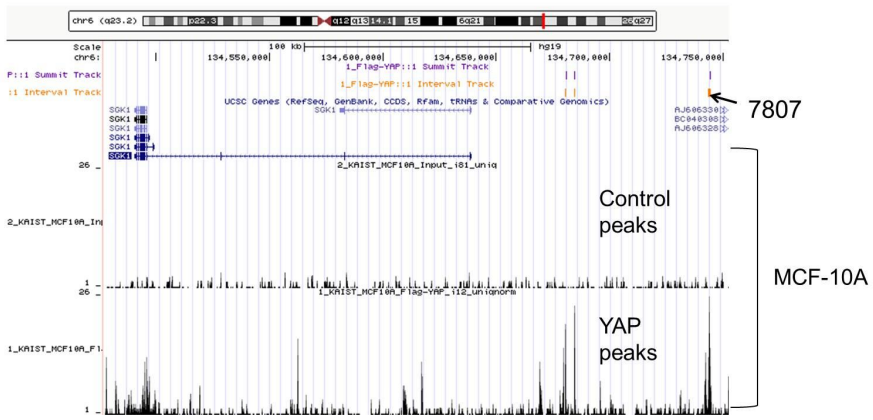
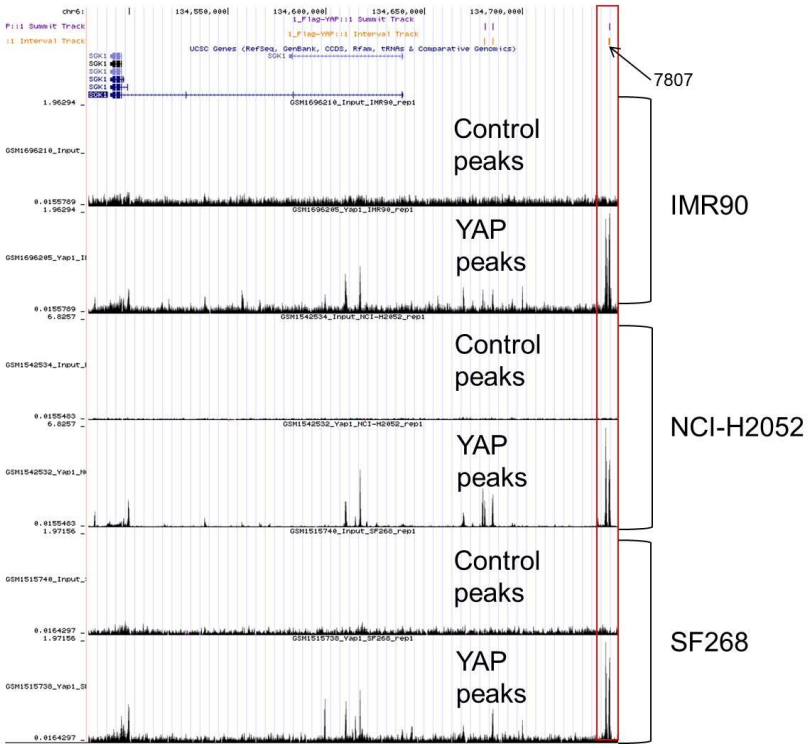

7807
Control peaks
YAP peaks
MCF-10A

Figure 1-4. Region "7807" is enriched by YAP in non-transformed and transformed cells.

YAP peak 7807 is correlated with YAP ChIP-seq peaks in IMR90, NCI-H2052, and SF268. YAP ChIP-seq data were downloaded from NCBI GEO (GSE61852) (Stein et al., 2015).

Then, YAP ChIP-seq data for each cell line were uploaded into UCSC genome browser. Orange rectangles indicate three YAP ChIP-seq peaks discovered from SGK1 regulatory sequence analysis of MCF-10A YAP ChIP-seq.


7807
Control peaks
YAP peaks
IMR90
Control peaks
YAP peaks
NCI-H2052
Control peaks
YAP peaks
SF268

total of four different YAP ChIP sequencing results indicate that not only YAP binds to SGK1 regulatory sequences but also that YAP peak 7807 may be specific region where YAP is potentially enriched.

As a next step, I decided to validate whether the peak 7807 is where YAP authentically binds to SGK1 regulatory sequences. First, I checked the reliability of our ChIP-qPCR analysis by confirming that YAP binds to the CTGF promoter region. As predicted, YAP bound to the CTGF promoter region as previously reported (Figure 1.5A) (Zhao et al., 2008). More importantly, our ChIP-qPCR indicated YAP directly bound to peak 7807 in the genome (Figure 1.5A). Interestingly, when I observed more closely the genomic sequences of YAP peak 7807, I found two DNA binding consensus sequences for TEAD, CATTCC (Figure 1.5B). This finding hints that YAP, which cannot directly bind to DNA, may bind to YAP peak 7807 genomic region by forming a complex with TEAD transcription factor. All these data are consistent with the hypothesis that YAP regulates SGK1 transcriptional upregulation by direct binding to SGK1 regulatory sequences with TEAD.

Next, I investigated whether TEAD transcription factor could bind to the putative SGK1 enhancer, 7807. When Bauer group's TEAD1 ChIP seq. (GSE61852) were analyzed, I found the strongest TEAD1 peak in region 7807 of the SGK1 regulator sequences, suggesting that both YAP and TEAD may cooperatively bind to the SGK1 enhancer, 7807 (Figure 1.6).

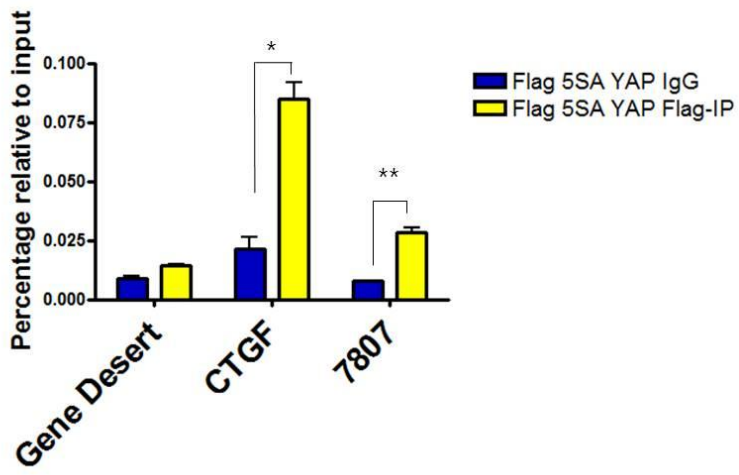
Since I found that both YAP and TEAD bind to SGK1 enhancer

Figure 1-5. YAP directly binds to region "7807."

(A) YAP ChIP-qPCR analysis of gene desert, CTGF promoter and 7807(the SGK1 enhancer) in 5SA YAP overexpressed MCF-10A. 5SA YAP was used for maximum YAP enrichment in the nucleus. Data are means ±SD; **P*<0.05; ***P*<0.01.

(B) Genomic sequences of the SGK1 enhancer region 7807. There are two TEAD consensus binding motif (CATTCC) in the sequence.

A

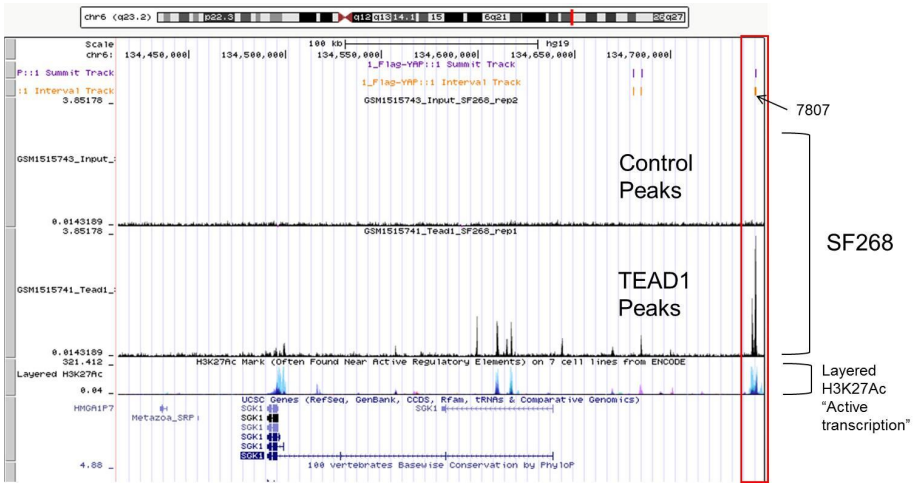


B 7807 genomic sequence

>hg19_dna range=chr6:134743519-134744451 5'pad=0 3'pad=0
strand=+ repeatMasking=none
CTGGTATCGAACTCCTGACCTCAGGTGATCCGCCTGCCTTGGCCTCCCAA
AGTGCTGGGATTACAGGCATGAGCCACTGTGCTCAGCCTCAATATCATAG
TAATTTAAAAAATTTAAGGCTACTTTTAACACAGAATAGCTAGTAATTTT
TTTTTAACTTTAACAGAACCAGAAGGTTACTCTGCACCAGGGAAAATGCA
TTTCCTCTCTCTCTTTCTACCTGTACCTCCCACTTCTG**CATTCC**ACTGCA
TTATTGGTCAAAGAAAAGCCAAATCTTTATATGGAATTTTTAACTCATTT
CCCAGTTATGCTTAGAATTCAAGATAGATAAACCTAGAGGCAATACAATT
CCCAAGTACGGTAAAAATAAAACAGAAGTACAGACAGCTATTAGTAAACA
CGTCTTCATCATGAGCTCAATTTCTTACCAAATGAAAATTTCCTCAATCA
CAGTGGTGAAAGTACTCAGCAATGGAGGCCTCA**CATTCC**ACACCAAAATG
AAAGAGACGGAATGCTTGTTTTTGTGGCGAAAAAAATCCCAACACTATTA
GTCACTTTTCTAACCAGATGGATAGAATGTTCCATGGTCTATAGATAATG
ATGACGACCATGAGTTGGTGAGTGACCTGTGAGATGAGTCAACTGGCGAA
GCAGTTGTTTTGACTACACCAAACCTAGGGAACTCAGATGGAAGTGGGTT
TGGTATTTTTCTGTCAGAGCTGCCACTTTTGGTGTTGGGAGGGTTCAGGA
AGAGGAAACTGAGAATAAGTGATTAGATAGATTTGTGGCCATGTCTGGGG
AAGACAGGGCAAGGCTGATGCTGTTGGGTGGGAGGTGAGGAAGGACTG
GGGCTGGCTTGCACACTCTTGTGTCTGCGTTAACCGTTGCTCTTCTTTTCTG
ACTGTAAAACACAAGTGAAAGTGTAGGGAAAAT

Figure 1-6. TEAD1 binds to region "7807."

TEAD1 ChIP-seq analysis of SF268 cell line. TEAD1 ChIP-seq data were downloaded from NCBI GEO (GSE61852) and then uploaded onto UCSC genome browser. The arrow indicates region 7807 identified from YAP ChIP-seq of MCF-10A that is also TEAD1 bound. Histine 3 Lysine 27 Acetylation (H3K27Ac) is a epigenetic marker of active transcription.


chr6 (q23.2)
Scale
chr6:
134,450,000
134,500,000
134,550,000
134,600,000
134,650,000
134,700,000
100 kb
hg19
1_Flag-YAP::1 Summit Track
1_Flag-YAP::1 Interval Track
GSM1515743_Input_SF268_rep2
7807
Control Peaks
GSM1515741_Tead1_SF268_rep1
TEAD1 Peaks
SF268
H3K27Ac Mark (Often Found Near Active Regulatory Elements) on 7 cell lines from ENCODE
Layered H3K27Ac
Layered H3K27Ac "Active transcription"
UCSC Genes (RefSeq, GenBank, CCDS, Rfam, tRNAs & Comparative Genomics)
100 vertebrates Basewise Conservation by PhyloP

region 7807, I wanted to test whether this binding is actually functional in YAP mediated SGK1 transcription. To do so, I designed SGK1 luciferase reporter construct that mimics the SGK1 genomic region. My firefly luciferase construct consisted of SGK1 promoter region near the SGK1 Transcriptional start site and YAP peak region 7807 located in the enhancer position placed behind the Luciferase (Figure 1.7A). If YAP really bound to the enhancer region of SGK1 and promotes SGK1 transcription by chromatin looping, my aforementioned SGK1 reporter construct should be responsive to YAP overexpression. To validate the above notion was actually reliable, I transfected hyperactive 5SA YAP or TEAD binding deficient 5SA94A with my SGK1 reporter construct. Upon overexpression of 5SA YAP, I found an approximately 3~4 fold increase in SGK1 reporter activity, consistent with the hypothesis that YAP controls SGK1 transcription by YAP binding to the SGK1 enhancer region 7807 (Figure 1.7B). Furthermore, consistent with my previous results, YAP that cannot bind to TEAD failed to activate the SGK1 reporter, signifying the importance of TEAD transcription factor in SGK1 transcription. These data support that YAP binding to SGK1 genomic region is a functionally significant event.

Next, I wanted to examine the functional significance of SGK1 transcription by YAP. YAP confers oncogenic property to cells upon overexpression, and therefore, I hypothesized that SGK1 transcription by YAP has an additive role in YAP's oncogenic function. Along this line, SGK1 was recently recognized to be important in

Figure 1-7. YAP increases SGK1 reporter activity in a TEAD-dependent manner.

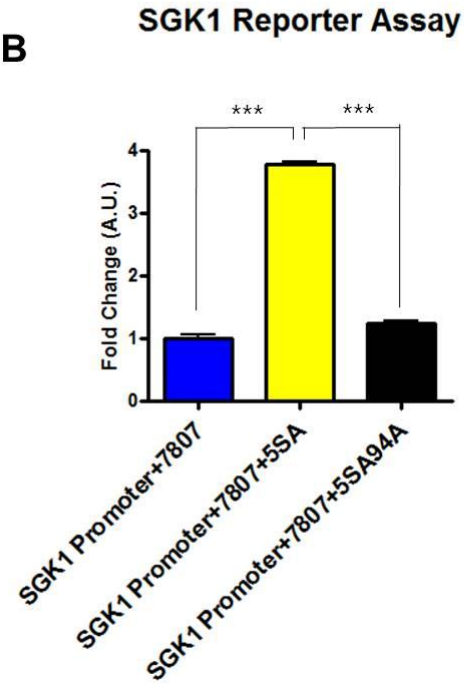
(A) Schematic diagram of SGK1 luciferase reporter mimicking SGK1 genomic region.

(B) SGK1 reporter markedly responded to 5SA YAP overexpression, but not in 5SA 94A YAP. SGK1 luciferase reporter constructs were transfected into HEK293 cells in combination with YAP mutants and renilla luciferase. Cells were harvested after 24 h and luciferase signals were measured by laminator. Firefly luciferase activity was normalized by Renilla luciferase. Data are means ±SD; ***P<0.001.

A


TSS
SGK1
Promoter
Luciferase
7807

B


SGK1 Reporter Assay
***
***
Fold Change (A.U.)
0
1
2
3
4
SGK1 Promoter+7807
SGK1 Promoter+7807+5SA
SGK1 Promoter+7807+5SA94A

anchorage-independent growth, which mimics metastatic cancer cell survival in unfavorable conditions (Mason et al., 2016). Thus, I predicted that both YAP and SGK1 may be functional for growth in harsh environments. In order to test this, I performed a soft agar assay using 5SA YAP or 5SA YAP with SGK1-depleted MCF-10A. As expected, SGK1 knockdown in YAP 5SA-overexpressing MCF-10A significantly reduced colony size (Figure 1.8). This result implies that SGK1 is required for anchorage-independent growth in YAP activated MCF-10A.

SGK1 enhances YAP/TAZ activity in MCF-10A cells.

While analyzing literature, I incidentally came across primary articles that indicated SGK1 controls CTGF, which is a well-known downstream target of YAP/TAZ (Vallon et al., 2006, Hussain et al., 2008, Zhao et al., 2008, Zhang et al., 2009). These reports hinted at the possibility of SGK1 controlling YAP/TAZ activity. Therefore, to investigate whether SGK1 can positively affect YAP/TAZ, I quantified levels of YAP/TAZ target genes upon SGK1 knockdown in MCF-10A. I chose CTGF and CYR61, since they are representative target genes of YAP and TAZ. When I measured mRNA levels of CTGF and CYR61, their levels were significantly reduced and positively correlated to SGK1 knockdown status (Figure 1.9A). Before performing gain of function experiments, I generated mutant SGK1 forms by nested PCR. SGK1 has two notable regulatory amino acid residues that are

Figure 1-8. SGK1 is required for anchorage-independent growth property of YAP over-expressing MCF-10A.

(A) Colony formation assay in MCF-10A cells stably expressing 5SA YAP with either shGFP or shSGK1. Magnified view of representative colonies in each type of stable cell line stained with crystal violet and imaged with a dissecting microscope.

(B) Quantification of average colony size and number of colonies for the results in (A). *P<0.05.

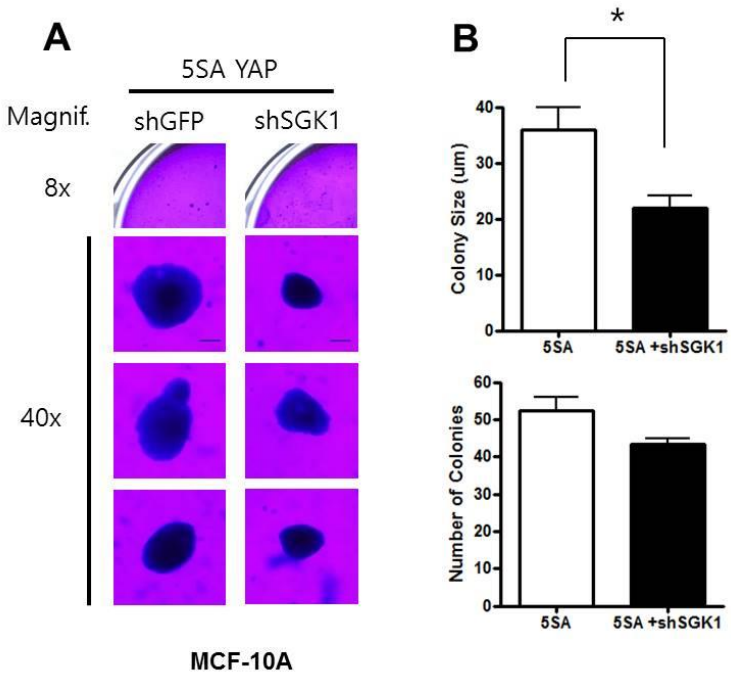
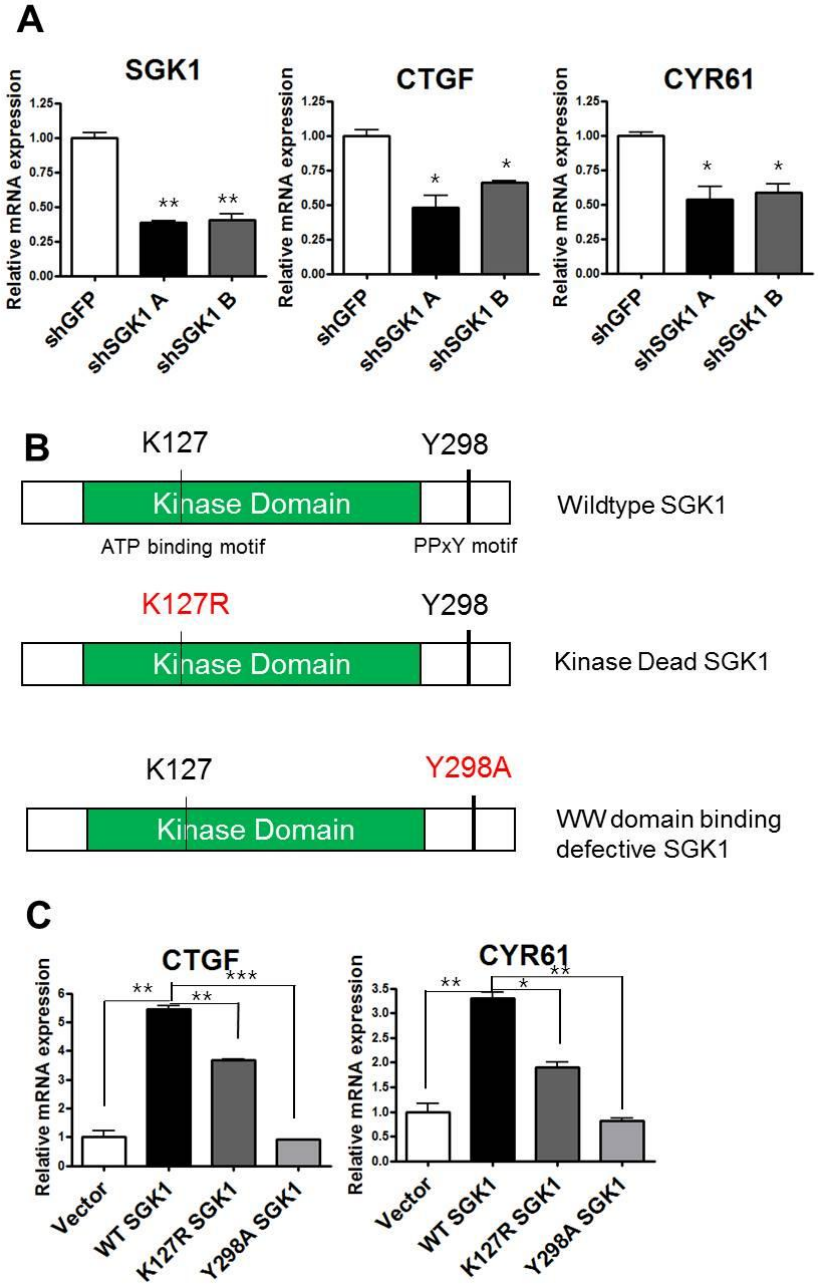

A
5SA YAP
Magnif.
shGFP
shSGK1
8x
40x
MCF-10A
B
*
Colony Size (um)
40
30
20
10
0
5SA
5SA +shSGK1
Number of Colonies
60
50
40
30
20
10
0
5SA
5SA +shSGK1

Figure 1-9. SGK1 enhances YAP/TAZ target genes, CTGF and CYR61.

(A) Quantitative PCR analysis of MCF-10A cells stably expressing control verses shSGK1. Data are means ±SD; *P<0.05; **P<0.01.

(B) Schematic of SGK1 mutants used in the study containing ATP binding motif and PY motif.

(C) Quantitative PCR analysis of MCF-10A cells stably expressing either vector or Flag-SGK1 (WT, K127R, and Y298A). Data are means ±SD; *P<0.05; **P<0.01. ***P<0.001.


A
SGK1
CTGF
CYR61
Relative mRNA expression
shGFP
shSGK1 A
shSGK1 B
B
K127
Y298
Kinase Domain
ATP binding motif
PPxY motif
Wildtype SGK1
K127R
Kinase Dead SGK1
Y298A
WW domain binding defective SGK1
C
Vector
WT SGK1
K127R SGK1
Y298A SGK1

important for its activity (Figure 1.9B). One is Lysine 127, which is the ATP-binding site of SGK1 kinase domain, and when mutated to arginine, SGK1 loses its kinase activity. Therefore, K127R site is critical for SGK1 kinase activity (Synder et al., 2002). The other site is Tyrosine 298, of its part of PPxY motif, which mediates protein-protein interaction with WW-domain containing proteins such as NEDD4 and NEDD4L E3 ubiquitin ligases (Synder et al., 2002). When this Tyrosine residue is substituted to Alanine, WW domain containing proteins lose affinity to SGK1, and therefore, SGK1 from its potential substrates. Then reciprocally, I decided to evaluate the transcript levels of CTGF and CYR61 in SGK1 over-expressed MCF-10A. Upon WT SGK1 overexpression, there was a significant increase in levels of CTGF and CYR61 which was positively correlated to SGK1 level while CTGF and CYR61 levels in SGK1 mutant overexpressing cells were significantly reduced compared to that of WT SGK1 (Figure 1.9C). From the above results, I can infer that SGK1 kinase activity and its binding affinity to WW domain-containing proteins may have a regulatory role in SGK1 control of YAP/TAZ target gene levels.

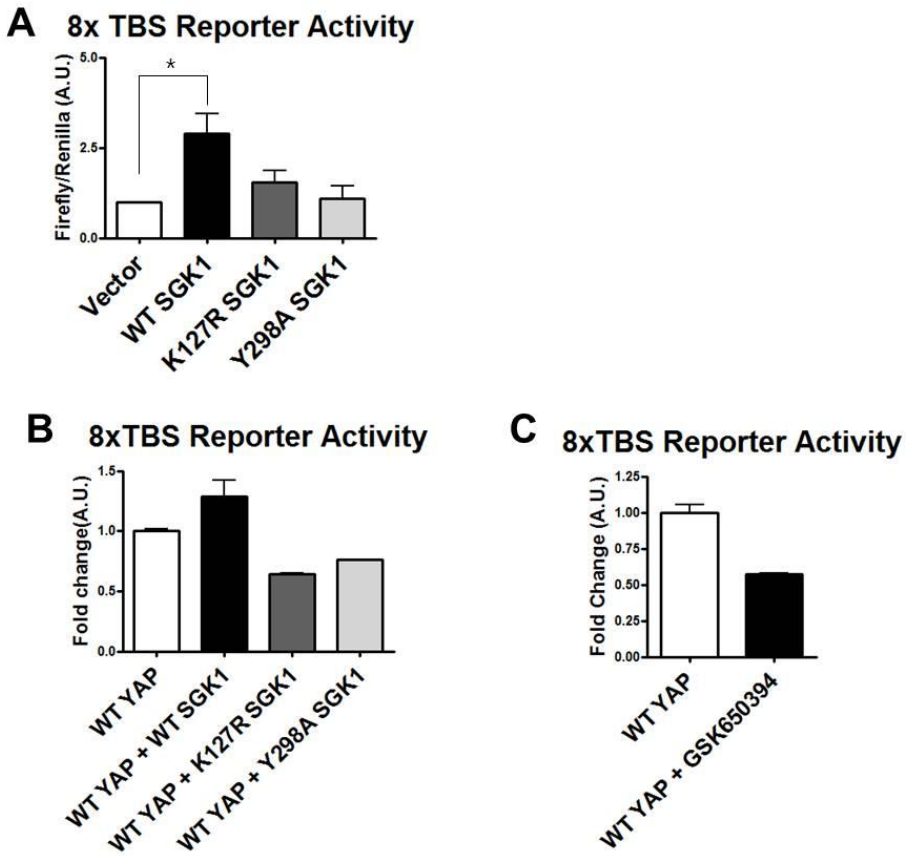
Next, to examine whether SGK1 controls activity of YAP-TEAD complex, I assessed 8x-TEAD binding sequence (TBS) reporter activities upon SGK1 overexpression in HEK293 cells. 8x-TBS reporter assay has previous been shown to be a reliable YAP-TEAD reporter (Kim et al., 2015a). Parallel to previous results, WT SGK1 overexpression increased YAP-TEAD activity while the activity was relatively reduced upon overexpression of either K127R SGK1 or Y298A SGK1 (Figure 1.10A).

Figure 1-10. SGK1 enhances YAP-TEAD reporter activity via its PPxY motif and Kinase domain.

(A) Dual Reporter Luciferase analysis of HEK293 cells transiently transfected with different forms of SGK1 (WT, K127R, Y298A). Three independent experiments were performed to detect 8x TBS reporter activity. **P*<0.05. Data are means ±SD.

(B) Dual Reporter Luciferase analysis of HEK293 cells transiently transfected in combination of WT YAP and various SGK1 constructs. Data are means ±SD.

(C) Dual reporter luciferase analysis of HEK293 cells transiently transfected with WT YAP and treated with SGK1 inhibitor, GSK650394. Data are means ±SD.


A
8x TBS Reporter Activity
Firefly/Renilla (A.U.)
5.0
2.5
0.0
*
Vector
WT SGK1
K127R SGK1
Y298A SGK1
B
8xTBS Reporter Activity
Fold change(A.U.)
1.5
1.0
0.5
0.0
WT YAP
WT YAP + WT SGK1
WT YAP + K127R SGK1
WT YAP + Y298A SGK1
C
8xTBS Reporter Activity
Fold Change (A.U.)
1.25
1.00
0.75
0.50
0.25
0.00
WT YAP
WT YAP + GSK650394

Furthermore, co-overexpression of WT YAP and WT SGK1 synergistically reinforced YAP-TEAD reporter activity compared to single overexpression of WT YAP (Figure 1.10B). Meanwhile, TEAD activities measured upon overexpression of SGK1 mutants (K127R or Y298A) confirmed that these residues are important for cooperation between YAP and SGK1 for YAP-TEAD activity (Figure 1.10B). Alternatively, I treated SGK1 inhibitor, GSK650394, on WT YAP overexpressed HEK293 cells. GSK650394 is a competitive small molecule inhibitor of SGK1 and has previously been shown to block prostate cancer cell growth (Sherk et al., 2008). As predicted, I confirmed that SGK1 activity was important for WT YAP induced increase in YAP-TEAD activity (Figure 1.10C). These data suggest that SGK1 kinase activity and SGK1 PPxY motif were important for SGK1 mediated synergism on YAP-TEAD activity.

SGK1 enhances YAP/TAZ activity by upregulation of YAP/TAZ in MCF-10A cells.

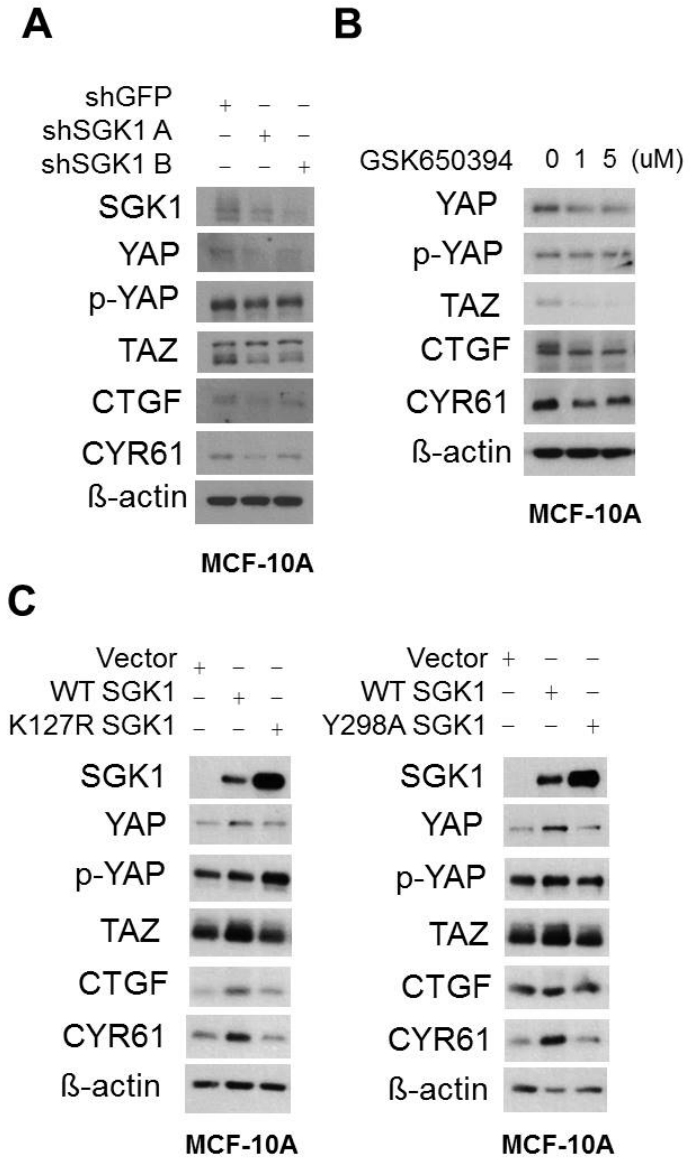
Knowing that SGK1 enhances transcriptional levels of CTGF and CYR61, I next analyzed the protein levels of CTGF and CYR61 upon SGK1 knockdown in MCF-10A. In concurrence with analysis of CTGF and CYR61 mRNA levels, CTGF and CYR61 protein levels were down-regulated upon SGK1 knockdown (Figure 1.11A). More surprisingly, when I observed the protein levels of YAP and TAZ, their levels were down-regulated and positively correlated with CTGF

Figure 1-11. SGK1 increases YAP and TAZ protein levels.

(A) MCF-10A cells each stably expressing shGFP or shSGK1 A or shSGK1 B was analyzed by Western blot with various antibodies.

(B) MCF-10A cells treated with GSK650394 (DMSO as a vehicle) for 24 h were analyzed by Western blot with various antibodies.

(C) MCF-10A cells each stably expressing Vector, WT SGK1, K127R SGK1, and Y298A SGK1 were analyzed by Western blot with various antibodies.


A
shGFP + – –
shSGK1 A – + –
shSGK1 B – – +
SGK1
YAP
p-YAP
TAZ
CTGF
CYR61
ß-actin
MCF-10A
B
GSK650394 0 1 5 (uM)
YAP
p-YAP
TAZ
CTGF
CYR61
ß-actin
MCF-10A
C
Vector + – –
WT SGK1 – + –
K127R SGK1 – – +
SGK1
YAP
p-YAP
TAZ
CTGF
CYR61
ß-actin
MCF-10A
Vector + – –
WT SGK1 – + –
Y298A SGK1 – – +
SGK1
YAP
p-YAP
TAZ
CTGF
CYR61
ß-actin
MCF-10A

and CYR61 levels (Figure 1.11A). This observation led us to hypothesize that SGK1 regulates YAP/TAZ to control their target genes. Interestingly, phospho-YAP (Ser127), a critical phosphorylation site for YAP activity and YAP cytoplasmic localization as previously reported (Zhao et al., 2008), was not upregulated by SGK1 knockdown, meaning that SGK1 mediated upregulation of YAP/TAZ is LATS kinase activity independent (Figure 1.11A). In order to confirm SGK1-mediated upregulation of YAP/TAZ, I treated SGK1 inhibitor, GSK650394 in MCF-10A and observed for changes in YAP/TAZ activity to SGK1 kinase inactivation. In agreement with SGK1 knockdown results, SGK1 inhibitor treatment led to not only reduction of CTGF and CYR61 levels, but also, reduction in YAP and TAZ (Figure 1.11B). Again, phospho-YAP (Ser127) was not significantly affected by SGK1 inhibitor, indicating that SGK1 mediated positive regulation of YAP/TAZ and their activities are independent from YAP phosphorylation on Serine 127. I then looked at the effects of SGK1 overexpression in YAP/TAZ and their target genes. Consistent with our previously shown transcript analyses, WT SGK1 increased CTGF and CYR61 protein levels while kinase dead K127R SGK1 and PPxY motif mutated Y298A SGK1 each decreased CTGF and CYR61 comparable to basal state levels (Figure 1.11C). As previously mentioned, phospho-YAP levels were not correlated with SGK1 levels, suggesting that SGK1 controls YAP irrespective of Serine 127 phosphorylation. Our previously shown loss of function and gain of function experiments uniformly indicate SGK1 as a positive regulator of YAP/TAZ and their

target genes.

In order to ensure that SGK1 upregulation of CTGF and CYR61 are YAP/TAZ-dependent, I ablated the levels of both YAP and TAZ in SGK1 overexpressing MCF-10A cells. When I analyzed both protein levels and mRNA levels of upon YAP/TAZ knockdown in SGK1 overexpressing set, CTGF and CYR61 were drastically reduced, implying that YAP/TAZ lie downstream of SGK1 in controlling levels of CTGF and CYR61 (Figure 1.12A). Interestingly, I also found that SGK1 overexpression marginally, but not significantly increased TAZ transcript, while slightly reducing YAP transcript (Figure 1.12B). This result suggests that SGK1 does not strongly affect YAP and TAZ transcripts. Therefore, I turned to investigate the mode of YAP and TAZ regulation by SGK1 at the post-translational level.

SGK1 stabilizes TAZ via GSK3B inhibition in MCF-10A cells.

In order to scrutinize SGK1 regulation of YAP/TAZ at the post-translational level, I treated MG132, a proteasomal inhibitor, in SGK1 knockdown MCF-10A cells. Upon treating MG132, due to inhibition of proteasome mediated protein degradation, I noticed a significant TAZ protein accumulation in SGK1 knockdown set comparable to control knockdown set, while in comparison, not much change was seen in that of YAP levels (Figure 1.13A). I reasoned the differences between YAP and TAZ in protein accumulation is due to fact that TAZ is a versatile protein with a protein half-life of

Figure 1-12. SGK1 enhances CTGF and CYR61 expression in a YAP/TAZ-dependent manner.

(A) MCF-10A cells stably expressing either vector or WT SGK1 were treated with either control siRNA or combination of siYAP and siTAZ (in 1:2 ratio) for 48h were analyzed by Western blot with various antibodies.

(B) MCF-10A cells stably expressing either vector or WT SGK1 treated with either control siRNA or combination of siYAP/siTAZ were analyzed by qPCR.

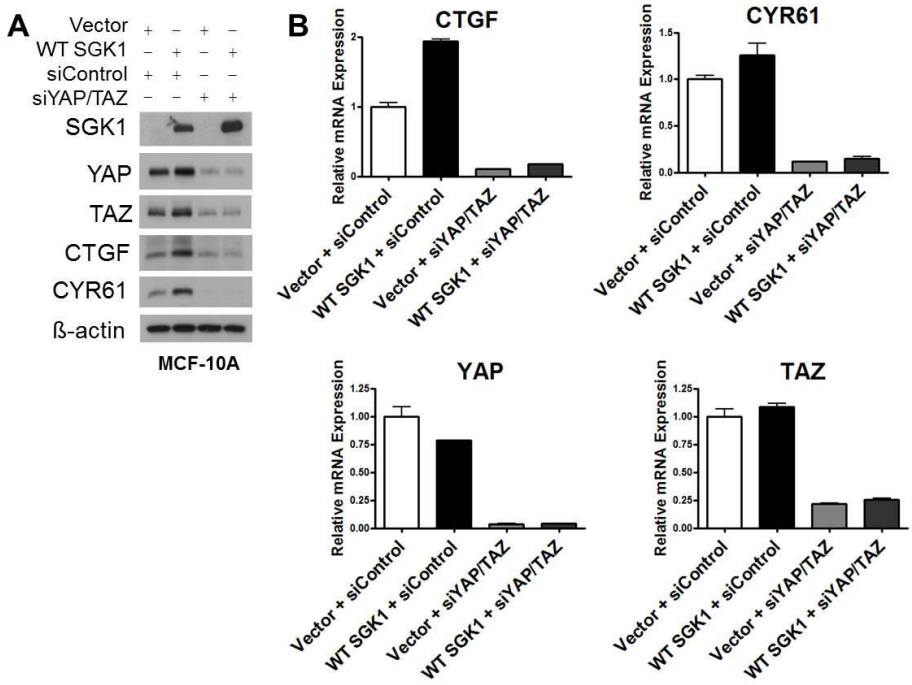
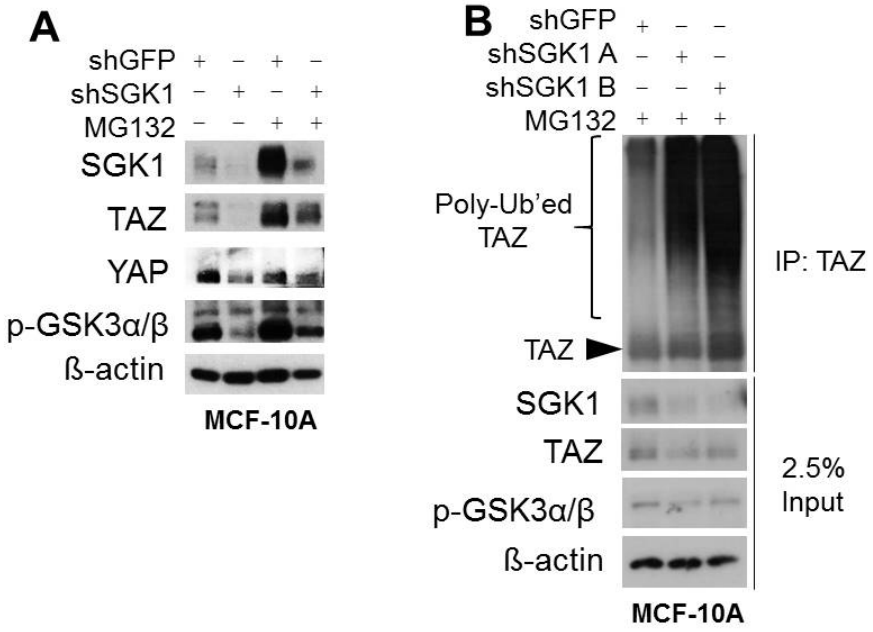

A
Vector + – + –
WT SGK1 – + – +
siControl + + – –
siYAP/TAZ – – + +
SGK1
YAP
TAZ
CTGF
CYR61
ß-actin
MCF-10A
B
CTGF
CYR61
YAP
TAZ
Relative mRNA Expression
Vector + siControl
WT SGK1 + siControl
Vector + siYAP/TAZ
WT SGK1 + siYAP/TAZ

Figure 1-13. SGK1 stabilizes TAZ protein.

(A) MCF-10A cells stably expressing either shGFP or shSGK1 were either treated vehicle (DMSO) or 50 μM MG132 for 6 h and analyzed by Western blot.

(B) MCF-10A cells stably expressing either shGFP or shSGK1 A or shSGK1 B was treated with 50 μM MG132 for 6 h and then *in vivo* ubiquitination assay was performed.


A
shGFP + − + −
shSGK1 − + − +
MG132 − − + +
SGK1
TAZ
YAP
p-GSK3α/β
ß-actin
MCF-10A
B
shGFP + − −
shSGK1 A − + −
shSGK1 B − − +
MG132 + + +
Poly-Ub'ed TAZ
TAZ
IP: TAZ
SGK1
TAZ
p-GSK3α/β
ß-actin
2.5% Input
MCF-10A

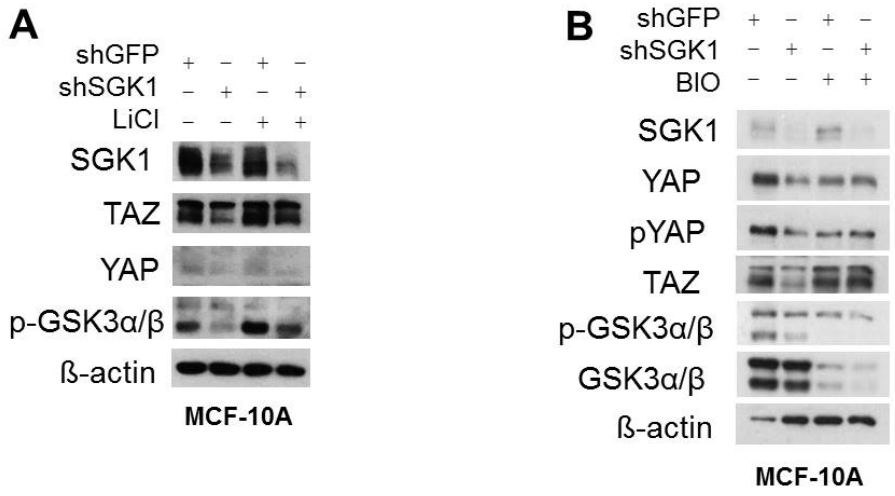
approximately 2h while YAP is relatively a stable protein with a half-life of 12h (Liu et al., 2010). Therefore, I decided to focus on SGK1 regulation of TAZ from this point on. Next, in order to confirm the post-translational regulation of TAZ by SGK1, I performed *in vivo* ubiquitination assay in MCF-10A cells that have both been silenced with shSGK1 and treated with MG132. In concurrence with the previous result in Figure 1.12A, SGK1 knockdown caused an increase in poly-ubiquitination of TAZ, suggesting SGK1 stabilizes TAZ from ubiquitin mediated protein degradation (Figure 1.13B). These data suggest that SGK1 controls TAZ at the post-translational level.

A recent study by Huang et al. suggested TAZ stability can be regulated by protein kinases other than LATS1/2 (Huang et al. 2012). In their study, Huang et al. suggested that GSK3β is capable of destabilizing TAZ, through phosphorylation of the N-terminal residues of TAZ (Ser-58 and Ser-62) that are absent in YAP (Huang et al. 2012). More interestingly, SGK1 is known to inhibit GSK3β by phosphorylation in Serine 9 residue of GSK3β (Cohen et al. 2001, Sakoda et al. 2003). In order to test the idea that SGK1 stabilizes TAZ by phosphorylating and inhibiting SGK1 substrate, GSK3β, I treated GSK3β inhibitors, Lithium Chloride or BIO in SGK1 knockdown cells (Figure 1.14A-B). Surprisingly, I found that both GSK3β inhibitors rescued the SGK1 knockdown phenotype, while BIO is more efficient in rescuing TAZ (Figure 1.14A-B). Therefore, I concluded that SGK1 enhances TAZ stabilization by phosphorylating and inhibiting GSK3β.

Figure 1-14. SGK1 stabilizes TAZ protein via SGK1-GSK3β-TAZ inhibitory circuit.

(A) MCF-10A cells stably expressing shGFP or shSGK1 were treated with GSK3B inhibitor, 25 mM LiCl for 24 h and later analyzed by Western blot.

(B) MCF-10A cells stably expressing shGFP or shSGK1 were treated with 8 μM BIO for 24 h and analyzed by Western blot.


A
shGFP + − + −
shSGK1 − + − +
LiCl − − + +
SGK1
TAZ
YAP
p-GSK3α/β
ß-actin
MCF-10A
B
shGFP + − + −
shSGK1 − + − +
BIO − − + +
SGK1
YAP
pYAP
TAZ
p-GSK3α/β
GSK3α/β
ß-actin
MCF-10A

Discussion

Here I report that SGK1 is a novel downstream target gene of YAP. I showed that SGK1 is rapidly upregulated by YAP in a manner that resembles SGK1 stimulation to serum. Moreover, SGK1 upregulation is dependent on YAP activation status and YAP's ability to bind to its transcription factor TEAD. Therefore, YAP's role in transcriptional activation of SGK1 is dependent on TEAD. In agreement with these data, previous works by Zhao et al. (Zhao et al., 2008) proposed a critical role for TEAD in YAP-mediated transcription of its target genes. However, even though it seems convincing that TEAD is important for SGK1 transcriptional regulation, I have not shown directly that TEAD binds to SGK1 enhancer region. Therefore, further study is necessary to be fully confident that TEAD transcription factor is required for YAP-mediated SGK1 transcription by chromatin looping. Also, epigenetic changes in the SGK1 enhancer, specifically region 7807 I identified in this study, should be taken into account as well. Recent papers by Stein et al. (Stein et al., 2015) revealed that distal enhancers controlled by YAP are often marked by H3K27 acetylation. Further work is required to confirm that the enhancer region 7807 is marked by H3K27 acetylation, mark associated with active transcription.

I also reported that reciprocally, SGK1 upregulates YAP and TAZ. In the case of TAZ, it has been shown by Huang et al. (Huang et al., 2012) that AKT overexpression elevated TAZ level in a dose-dependent manner. Since SGK1 and AKT are reported to have

functional redundancies due to overlapping kinase substrates, it seems plausible to predict that SGK1-mediated molecular phenomenon on YAP/TAZ are also valid by AKT. In fact, GSK3β is also substrate for AKT. I believe that knocking down both AKT and SGK would result in a more drastic phenotype on TAZ stability. In agreement with this notion, AKT and SGK1 are homologs of *Drosophila Akt*, which has been shown to upregulate YAP and TAZ homologs of *Drosophila Yorkie* (Ye et al., 2012).

Here, I found that the PPxY motif of SGK1 is important for upregulation of YAP and TAZ. Compared to the extent of importance stressed on SGK1's kinase activity, a role of SGK1 PPxY motif is often overlooked in this field. Recent studies in the Hippo pathway supports the importance of protein-protein interaction between PPxY motif and WW domains in mediating cellular phenotypes in both Hippo pathway dependent and independent manners (Zhao et al., 2010b, Chan et al., 2011). It seems possible that SGK1 may control YAP/TAZ activity through its PPxY motif. Further work is required on interaction partners of SGK1 in relevance to the Hippo pathway.

CHAPTER II

Negative regulation of TAZ by YAP

Abstract

YAP and TAZ are critical effectors of Hippo signaling pathway that promote proliferation and cell survival. Although YAP and TAZ are commonly regulated by their upstream regulators LATS1/2, they have differences in physiological outcomes indicated by their knock-out phenotype in mouse models. Here, we report that YAP regulates TAZ in a unidirectional mechanism in various cell lines, including normal and cancer cells. Utilizing loss of function approaches, we also show that TAZ regulation by YAP is not regulated at the transcriptional level and post-translational level, but at the translational level. Lastly, we show that YAP negative regulation of TAZ is mediated in a Hippo pathway-independent manner, implying existence of a novel regulatory relationship between YAP and TAZ.

Introduction

Hippo signaling pathway was originally established in *Drosophila melanogaster*. Hippo pathway consists of a kinase cascade including Hippo-Warts, and they are found to enhance cell proliferation while suppressing apoptosis (Wu et al., 2003). *Yorkie* (*Yki*) was later found to be the downstream effector molecule of the Hippo pathway (Huang et al., 2005). *Yki* is phosphorylated by Warts kinase to allow precise binding for 14-3-3. *Yki*'s binding to 14-3-3 in turn translocates Yki to the cytoplasm, thus blocking its nuclear function (Dong et al., 2007). The mammalian homologs of *Yki* are YAP and TAZ. In this sense, YAP and TAZ are paralogs to each other. YAP and TAZ are similarly phosphorylated by LATS1/2 kinase, the mammalian homolog of *Warts*, to be sequestered from the nucleus by 14-3-3. As foreseen by the dramatic phenotype of *Yki*-overexpressing flies which exhibit massive overproliferation of imaginal discs, YAP and TAZ were also found to be potent oncogenes in mammals. YAP overexpression promotes hyperproliferation, growth factor independence, Epithelial-Mesenchymal Transition (EMT), resistance to apoptosis, and ability to form anchorage independent colonies in soft agar, which are major hallmarks of cancer (Overholtzer et al., 2006, Hanahan et al., 2011). Also, YAP overexpressing transgenic mice exhibit rapid and marked hypertrophy particularly in the liver and intestine (Camargo et al., 2007). Finally, YAP overexpression and nuclear localization of YAP was frequently found in many cancers (Steinhart et al., 2008).

Therefore, YAP is emerging as an important oncogene that regulates multiple aspects of tumorigenesis both in *Drosophila* and mammals.

TAZ, like YAP, was also initially identified as a 14-3-3 binding protein. TAZ has approximately 40~50% sequence similarity to YAP, and has common features and domains, namely the WW domain, PDZ binding motif, and TEAD binding motif. As described above, YAP regulation by Hippo upstream kinases is also conserved in TAZ (Zhao et al., 2008b). With regards to tumorigenesis, TAZ is highly expressed in various types of cancers, promotes cancer invasion, epithelial to mesenchymal transition, and anchorage- independent growth (Chan et al., 2008). Therefore, YAP and TAZ function analogously during the tumorigenesis.

However, YAP and TAZ have obvious differences in mouse model phenotypes. While YAP knockout mouse is embryonic lethal (Morin-Kensicki et al., 2006), TAZ knockout mouse is relatively healthy except with some problems in the kidney (Hossain et al., 2007). In addition, YAP and TAZ have distinct structural features as well. While YAP2 isoform has two WW domains, TAZ has only one WW domain. YAP has a proline-rich domain in the N-terminus while TAZ does not. TAZ has N-terminal phosphodegron targeted by CK1 and GSK3β, which YAP lacks (Huang et al., 2012). YAP has a SH3 binding motif with which it binds to Yes oncoprotein while TAZ lacks such a binding motif. Thus, even though YAP and TAZ are both subject to Hippo signaling mediated regulation, there is still a need for a further study between YAP and TAZ.

In this study, we found that YAP regulates TAZ in non-transformed and transformed cells. We show that YAP regulation of TAZ is neither transcriptional nor post-translational, but rather translational. More interestingly, YAP regulation of TAZ was found to be independent from the canonical Hippo pathway.

Materials and Methods

Cell Culture

MCF-10A was cultured incompleted mediumcontaining DMEM/F12 media supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, and 10 μg/ml insulin. RPE was cultured in DMEM/F12 media supplemented with 10% FBS.

293T and MDA-MB-231 were cultured in DMEM supplemented with 10% FBS. MEF (Mouse Embryonic Fibroblast) was cultured in DMEM supplemented with 10% FBS and L-Glutamine 200 μM. All of these media contained antibiotics (penicillin/streptomycin).

For drug treatment, the following compound was used: Cycloheximide (100 μM)

Western blot

Cells were harvested and lysed with RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) containing protease inhibitors and phosphatase inhibitors (1 μg/ml Pepstatin A, 1 μg/ml Leupeptin, 1 mM Phenylmethylsulfonyl Fluoride, 1 mM Sodium Orthovanadate, 5 mM Sodium Fluoride). Western blot analyses were performed using a standard protocol.

Antibodies

Antibodies used for western blot analysis include: YAP (raised against the C-terminal human YAP antigen by Kim et al. (Kim et al., 2015c)),

TAZ (Cell Signaling Tech.), ß-actin (Sigma), LATS1 (Cell Signaling Tech.), LATS2 (Cell Signaling Tech.), and pLATS1(S1079) (Cell Signaling Tech), MST1 (Cell Signaling Tech.), MST2 (Cell Signaling Tech.), and CTGF (Santa Cruz Biotech.).

siRNA and sgRNA

Lipofectamine RNAiMAX Reagent (Life Technologies) was used for transfection of siRNA. siRNA was prepared and transfected as described by the manufacturer. RNA oligonucleotides were synthesized by Samchully Pharmaceutical Co. siRNA sequences used in this study are siControl (GL2) (CGT ACG CGG AAT ACT TCG A), siYAP (GACAUCUUCUGGUCA GAGA), siTAZ (ACGUUG ACUUAGGAACUUU). siYAP and siTAZ sequences were obtained from Kim et al. (Kim et al. 2015b).

For generation of Knockout cell lines, following sequences were used to clone into the lentiCRISPRv2 puro plasmid (Addgene #52961) by HaeYon Jeon of Dr. Dae-Sik Lim's Lab:
sgLATS2 Forward (CACCGGTAGGACGCAAACGAATCGC), sgLATS2 Reverse (AAACGCGATTCGTTTGCGTCCTACC), sgLATS1 Forward (CACCGGCAACCTAACATACCAGTG), sgLATS1 Reverse (AAACCACTGGTATGTTAGGTTGCC).

After successful cloning, lentiviral sgLATS1 and sgLATS2 were transfected with viral packaging/capsid DNA to produce lentivirus. After that, lentiviruses were co-infected to MCF-10A and selected with puromycin for isolation of LATS1/2 double knockout single clone.

Quantitative PCR

RNA preparation and cDNA synthesis were done as described by the manufacturer using RiboEx (GeneAll) and M-MLV reverse transcriptase (Enzynomics). Quantitative polymerase chain reaction (qPCR) was performed using a SYBR green premix reagent (TOPreal qPCR 2X PreMIX; Enzynomics) and Bio-Rad CFX Connect instrument. Results were analyzed using Microsoft Excel.

Primers used for quantitative PCR are following:

hBeta-actin Forward (CATGTACGTTGCTATCCAGGC), hBeta-actin Reverse (CTCCTTAAT GTCACGCACGAT) hYAP Foward (GAACCAGAGAATCAGTCAGA), hYAP Reverse (GGATTGATATTCCGCATTGC), hTAZ Forward (GTCCTACGACGTGACCGAC), and hTAZ Reverse (CACGAGATTTGGCTGGGATAC), mGapdh Forward (AGGTCGGTGTGAACGGATTTG), mGapdh Reverse (TGTAGACCATGTAGTTGAGGTCA), mYap Forward (TACTGATGCAGGTACTGCGG), mYap Reverse (TCAGGGATCTCAAAGGAGGAC), mTaz Forward (CATGGCGGAAAAAGATCCTCC), mTaz Reverse (GTCGGTCACGTCATAGGACTG).

Metabolic labeling with radioactive Methionine

The day before the experiment, MCF-10A cells were transfected with either si-Control or si-YAP. The night before the experiment, each

MCF-10A cells were split so that cells will be confluent (1~2x10^7) on the day of experiment. On the experiment day, cells were pulse labeled with radioactive [^{35}S]Methionine (100 μCi) containing MCF-10A media (with dialyzed 5% horse serum and DMEM without L-Methioine and L-Cysteine) containing media supplements for 30 min on 37°C chamber. Pulse labeling was processed following a standard protocol (Bonifacino et al. 2002). Immediately after collecting cell pellets, cells were lysed with RIPA buffer and protein amounts were quantitated. 1 μg of anti-TAZ antibody (Santa Cruz Biotech) and 20 μl of Protein A/G agarose (GenDEPOT) were used for TAZ immunoprecipitation.

Results

TAZ is upregulated upon YAP depletion in normal and cancer cells.

We began to investigate the relationship between YAP and TAZ based on an unintended observation that TAZ was upregulated in YAP depleted cells. In MCF-10A and RPE1 cells that are considered to be normal cells, we observed a significant upregulation of TAZ protein level upon YAP knockdown, while a significant change in YAP level was not seen upon TAZ knockdown (Figure 2.1A-B). Such a Molecular phenotype of TAZ increase by YAP depletion was also apparent in MDA-MB-231, a malignant breast cancer cell line (Figure 2.1C). Moreover, a similar pattern was observed in mouse embryonic fibroblast in which Yap was knocked-out (Figure 2.1D). Interestingly, we observed a similar pattern of TAZ increase upon YAP knockdown in HeLa, MCF-7 and mesenchymal stem cells (Data not shown). Therefore, unidirectional YAP regulation of TAZ phenotype was a wide-spread molecular phenomenon than initially anticipated.

TAZ upregulation upon YAP depletion is not regulated at the transcriptional level.

Next, we pursued on investigating whether YAP negates TAZ at the transcriptional level, by measuring the relative TAZ mRNA expression upon YAP depletion. We found that TAZ expression was not significantly affected by YAP knockdown in MCF-10A, although YAP was not effectively knocked-down (Figure 2.2A). To be sure whether

Figure 2-1. TAZ is upregulated in YAP depleted cells.

(A) MCF-10A cells were transfected with either si-Control, si-YAP, or si-TAZ for 24h and analyzed by Western blot.

(B) RPE1 cells were transfected with corresponding siRNAs as in (A), and analyzed by Western blot.

(C) MDA-MB-231 cells were transfected with corresponding siRNAs as in (A-B), and analyzed by Western blot.

(D) Yap flox/flox Taz flox/+ and Yap flox/+ Taz flox/flox mouse embryonic fibroblasts were infected with control or Cre-expressing retrovirus for 48h and then selected with antibiotics, followed by Western blot analysis.

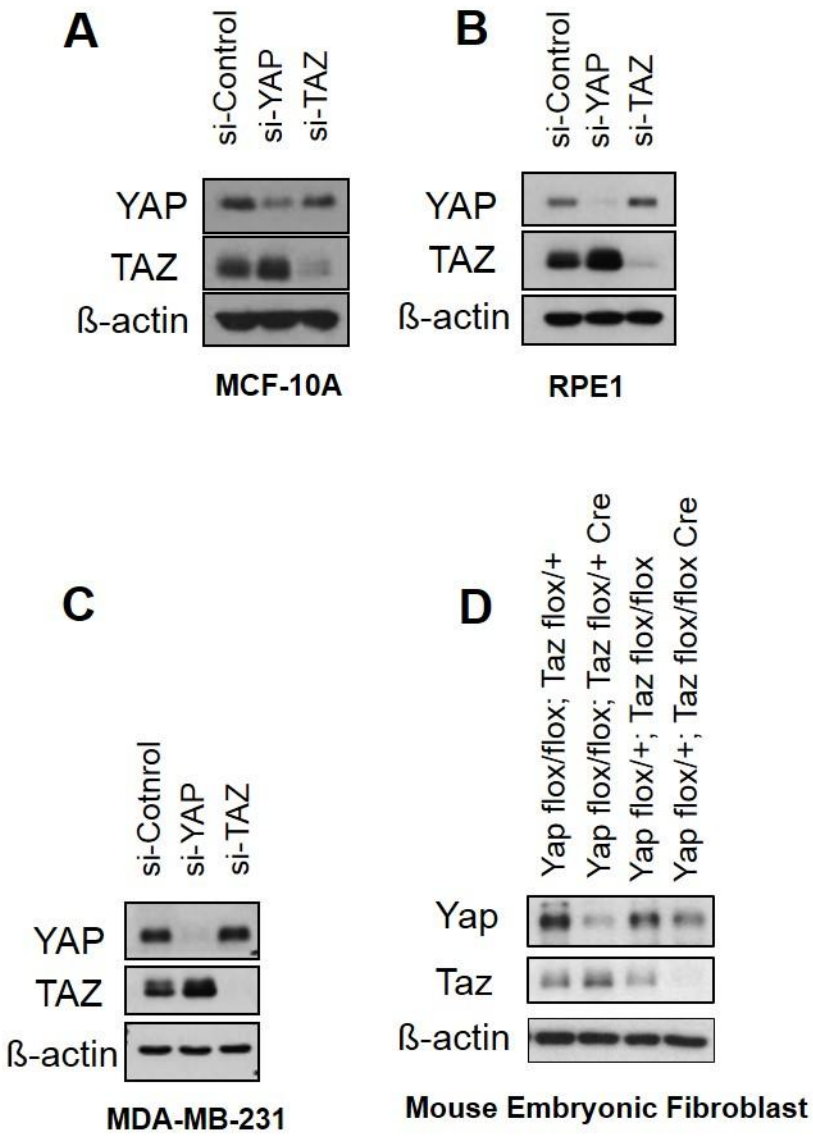

A
si-Control
si-YAP
si-TAZ
YAP
TAZ
ß-actin
MCF-10A
B
si-Control
si-YAP
si-TAZ
YAP
TAZ
ß-actin
RPE1
C
si-Cotnrol
si-YAP
si-TAZ
YAP
TAZ
ß-actin
MDA-MB-231
D
Yap flox/flox; Taz flox/+
Yap flox/flox; Taz flox/+ Cre
Yap flox/+; Taz flox/flox
Yap flox/+; Taz flox/flox Cre
Yap
Taz
ß-actin
Mouse Embryonic Fibroblast

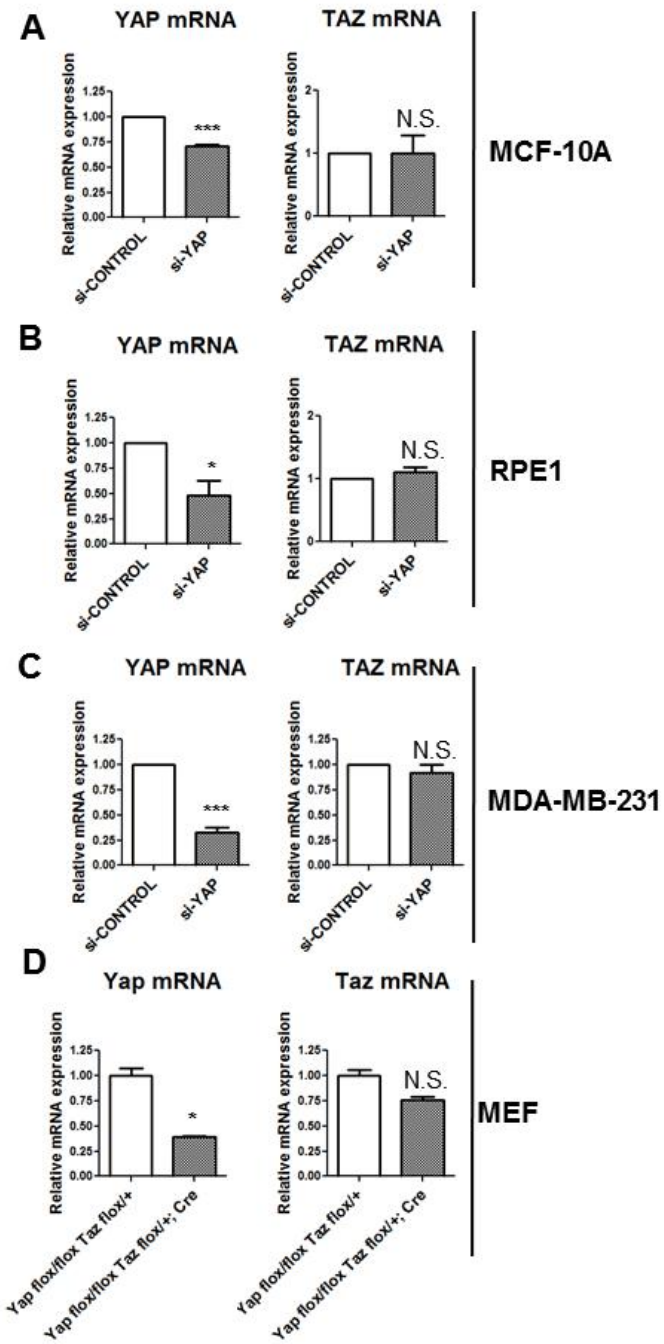
Figure 2-2. TAZ upregulation upon YAP knockdown is not significantly controlled at transcriptional level.

(A) MCF-10A cells were transfected with either si-Control or si-YAP for a day and then YAP and TAZ transcripts were quantitated by qPCR. (n=3)

(B) RPE1 cells were transfected with same siRNAs as in (A), and corresponding mRNAs were quantitated by qPCR. (n=3)

(C) MDA-MB-231 cells were transfected with same siRNAs as in (A-B), and then YAP and TAZ mRNAs were quantitated by qPCR. (n=3)

(D) Yap flox/flox Taz flox/+ MEF were treated with either control or Cre virus for 48h and then selected with puromycin. After that, Yap and Taz mRNAs were analyzed by qPCR. Data are means ±SD;*$p<0.05$; **$p<0.01$; ***$p<0.001$; N.S. Not statistically significant.


A
YAP mRNA
TAZ mRNA
Relative mRNA expression
1.25
1.00
0.75
0.50
0.25
0.00
***
N.S.
si-CONTROL
si-YAP
MCF-10A
B
YAP mRNA
TAZ mRNA
*
N.S.
RPE1
C
YAP mRNA
TAZ mRNA
***
N.S.
MDA-MB-231
D
Yap mRNA
Taz mRNA
*
N.S.
Yap flox/flox Taz flox/+
Yap flox/flox Taz flox/+; Cre
MEF

the TAZ phenotype we saw in MCF-10A was reproducible in other cell lines, we checked TAZ mRNA expression changes upon YAP knockdown in RPE1 and MDA-MB-231 cells. As expected, we obtained similar results in TAZ mRNA levels in YAP-depleted RPE1 and MDA-MB-231 cells (Figure 2.2B-C). Furthermore, Yap flox/flox Taz flox/+ MEF stably expressing Cre (which is virtually same as Yap Knockout), showed a consistent result as previous figures in that YAP does not significantly affect TAZ at the transcription level (Figure 2.2D). Interestingly, we realized that there are several papers published that supports our finding. For example, Xin et al. (Xin et al., 2013) showed that Yap conditional knockout heart does not lead to a statistically significant change in Taz mRNA expression. On the other hand, our result is surprising, considering the other group's report that overexpression of YAP reduces TAZ mRNA in a TEAD-dependent manner (Moroishi et al., 2015). A possible discrepancy may arise due to differences between loss of function and gain of function experiments. Therefore, YAP regulation of TAZ is, at least in the loss of function context, not significantly regulated at the transcriptional level.

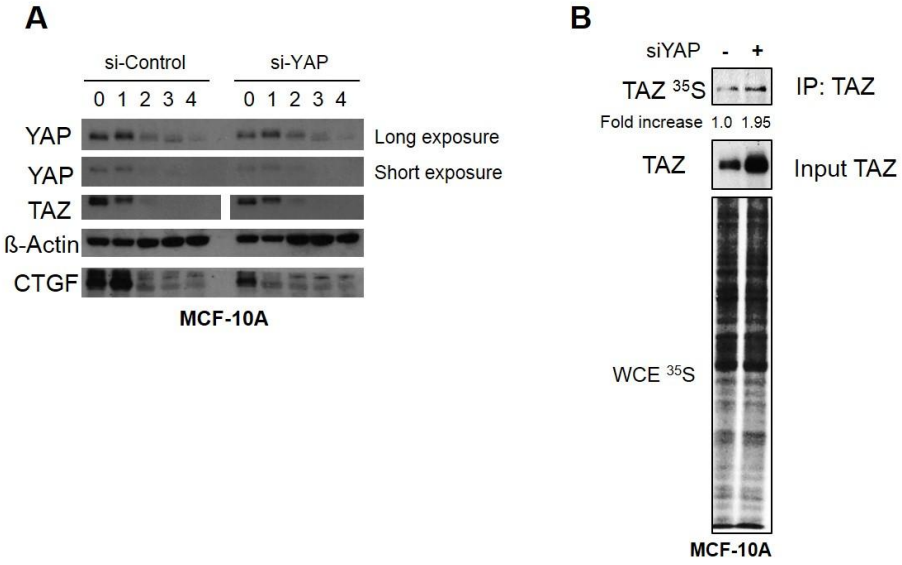
TAZ upregulation upon YAP knockdown is regulated at the translational level.

Next, we wanted to analyze at which level in central dogma of molecular biology, YAP negatively regulates TAZ. In order to test whether YAP regulates TAZ at the post-translational level, we

Figure 2-3. TAZ upregulation upon YAP depletion is not mediated by TAZ stabilization, but rather, in control of TAZ translation.

(A) Control or YAP knockdown MCF-10A cells were chased with Cycloheximide for indicated time points. ß-actin loading was adjusted to detect approximately equal amounts of TAZ at t = 0.

(B) Control or YAP knockdown MCF-10A cells were metabolically labelled for 30min and quantitated by image J.


A
si-Control
si-YAP
0 1 2 3 4
0 1 2 3 4
YAP
YAP
TAZ
ß-Actin
CTGF
Long exposure
Short exposure
MCF-10A
B
siYAP - +
TAZ 35S
IP: TAZ
Fold increase 1.0 1.95
TAZ
Input TAZ
WCE 35S
MCF-10A

performed cycloheximide chase experiment in control and YAP knocked-down cells. Cycloheximide is a translational inhibitor commonly utilized to acquire information about protein half-life. Thus, if YAP knockdown results in TAZ protein stabilization, one would expect elongated half-life of TAZ. However, the result was that there was virtually no difference in TAZ between control and YAP knocked-down sets (Figure 2.3A). Therefore, YAP seems to regulate TAZ neither at the transcriptional, nor post-translational level. Now, since we are left with translational level control to test, we performed metabolic pulse chase experiment with si-control and si-YAP transfected MCF-10A to investigate whether YAP regulates biosynthesis of TAZ protein. We chose Methionine as a radiolabeled amino acid of choice, because TAZ protein contains a sufficient number of Methionine (18 out of 404 amino acids; roughly 4.5 % of amino acid composition). When we performed the metabolic labeling experiment, we found that in YAP knockdown cells, TAZ biosynthesis rate was increased by approximately 1.95 fold (Figure 2.3B). Thus, we concluded that TAZ increase in YAP depletion was due to acceleration of TAZ biosynthesis.

TAZ up-regulation upon YAP depletion is regulated independent of Hippo signaling pathway.

Lastly, we examined YAP regulation of TAZ in the context of Hippo pathway. Since MCF-10A is considered to be one of the model cell lines useful for examining Hippo components, we focused our mechanistic study in MCF-10A. When we knocked-down YAP or TAZ

in MCF-10A and observed for any change in core Hippo kinase components, MST1/2 and LATS1/2, there was no obvious change in their amounts or activity (Figure 2.3A). Hypothetically speaking, if YAP negatively regulates TAZ through their upstream kinase LATS1/2, we expected a reduction in LATS1/2 activity represented by reduction in Serine 1079 phosphorylation. However, there was no change in LATS1/2 activity. In contrast, protein amounts of LATS1 and 2 were elevated in either YAP or TAZ knocked-down cells (Figure 2.4A). In order to distinguish whether YAP regulation of TAZ is only evident in certain context, we observed TAZ upregulation by YAP knockdown in Hippo pathway active verses inactive context represented by high versus low cell density, respectively. Intriguingly, YAP knockdown resulted in an increase of TAZ protein levels in both high and low density conditions, indicating Hippo pathway independent regulation of TAZ by YAP (Figure 2.4B). In order to validate that canonical Hippo pathway regulation of TAZ is dispensible for YAP regulation of TAZ, we knocked-out both LATS1 and LATS2 in MCF-10A by lentiCRISPR system. We found that when YAP is knocked-down in LATS1/2 knockout context, TAZ-upregulation by YAP-knockdown phenotype was still functional, shown by a marked increase in TAZ protein level (Figure 2.4C). Moreover, when 5SA YAP was overexpressed in LATS1/2 Knockout context, we still observed downregulation of TAZ by YAP overexpression (Figure 2.4D). In conclusion, TAZ regulation by YAP is mediated by a canonical Hippo pathway independent mechanism.

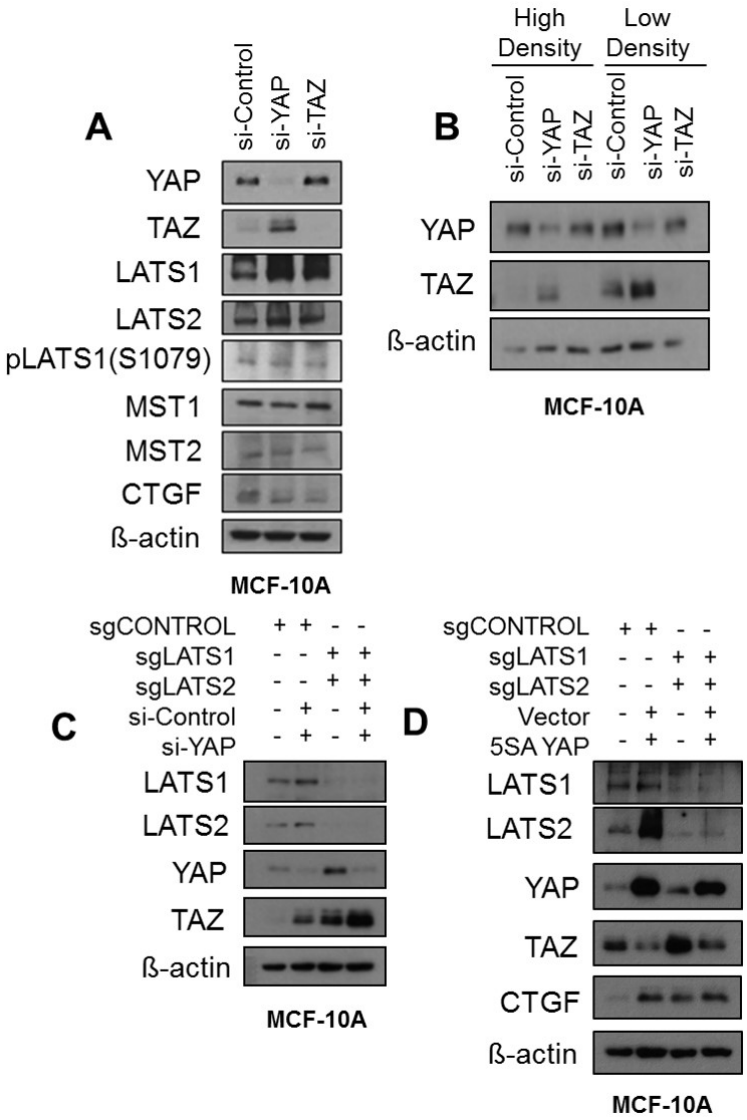
Figure 2-4. TAZ upregulation upon YAP knockdown is not mediated by Hippo signaling pathway.

(A) MCF-10A cells were transfected with indicated siRNAs for 24h and then cells were harvested for Western blot analysis with antibodies against Hippo related proteins.

(B) MCF-10A cells were transfected with same siRNAs as in (A), and then split into varying densities. After overnight incubation, cells were analyzed by Western blot.

(C) Stable cells of control or LATS1/2 Knockout were either transfected with si-Control or si-YAP for 24h and then analyzed by Western blot.

(D) Western blot analysis of control and LATS1/2 Knockout cells either stably expressing Vector or 5SA YAP using various antibodies.


A
si-Control
si-YAP
si-TAZ
YAP
TAZ
LATS1
LATS2
pLATS1(S1079)
MST1
MST2
CTGF
ß-actin
MCF-10A
B
High Density
Low Density
si-Control
si-YAP
si-TAZ
si-Control
si-YAP
si-TAZ
YAP
TAZ
ß-actin
MCF-10A
C
sgCONTROL + + - -
sgLATS1 - - + +
sgLATS2 - - + +
si-Control - + - +
si-YAP - + - +
LATS1
LATS2
YAP
TAZ
ß-actin
MCF-10A
D
sgCONTROL + + - -
sgLATS1 - - + +
sgLATS2 - - + +
Vector - + - +
5SA YAP - + - +
LATS1
LATS2
YAP
TAZ
CTGF
ß-actin
MCF-10A

DISCUSSION

Here, we found that TAZ is negatively regulated by its paralog, YAP. We also found that although YAP regulates TAZ, TAZ does not regulate YAP, meaning a unidirectional regulation exists between YAP and TAZ. Actually, while we were mid-way through this project, a paper by Finch-Edmondson et al., (Finch-Edmondson et al., 2015) reported the same observation of TAZ regulation by YAP in a unidirectional mechanism. Therefore, our observation was confirmed to be consistent and reproducible by other researchers. However, another group of scientists reported that YAP and TAZ bi-directionally regulate each other (Moroishi et al., 2015). In their study, Moroishi et al. utilized a gain of function approach to persuasively show that hyperactive YAP overexpression represses TAZ while hyperactive TAZ overexpression repressed YAP in cell culture systems. Meanwhile, Finch-Edmondson et al. utilized wildtype TAZ using an inducible overexpression system to show that YAP levels were unaffected.

Next, we have discovered that YAP does not regulate TAZ at the transcriptional level. To confirm this, we used multiple types of cell lines, including non-transformed and transformed, of human and mouse. And we consistently found that levels of human TAZ mRNA or mouse Taz mRNA were not significantly

affected by human YAP knockdown or mouse Yap knockout, respectively. These results are in sharp contrast to the report by Moroishi et al., in which they show hyperactive YAP repression of TAZ is abolished by mutation of the TEAD binding site to a defective form. There is certainly an issue concerning gain of function and loss of function experiments, in which dramatic changes in cellular environment may result incongruous cellular outcomes. Therefore, further study is required to clearly elucidate such context-dependent changes in regulatory relationship between YAP and TAZ.

Moreover, in contrast to Moroishi et al.'s claim that YAP regulation of TAZ is a Hippo dependent phenomenon, we obtained an opposite result which shows that TAZ regulation of YAP occurs independent from LATS1/2. One obvious difference between their result and ours is that they used mouse embryonic fibroblasts while we used MCF-10A cells as a model system to knock out LATS1/2. Further work is required to untangle this species-specific differences in the LATS1/2 KO phenotype.

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국 문 초 록

갈수록 복잡해지는 암 생물학계에서 최근 관심을 끄는 YAP과 TAZ 의 역할들을 볼 때, YAP과 TAZ의 주변 단백질 네트워크를 더욱 명확히 연구하는 것이 요구된다. 다시 말하면, 이미 잘 알려진 Hippo 신호전달체계에서의 YAP/TAZ 관련 세부적 조절 기작들을 밝히는 것이 YAP과 TAZ의 암 발달에서의 역할 정도를 이해하는 데에도 도움이 된다.

그러므로 나는 YAP과 TAZ 의 후보 조절인자들에 대한 연구를 진행하였다. SGK1은 PI3-K 신호전달체계의 종양단백질로서, AKT 억제제에 저항성을 띄는 암세포에서 과발현되는 중요한 타겟 유전자로 최근에 알려졌다. 나는 SGK1이 YAP의 활성도에 빠른 반응도를 가진 YAP의 타겟 유전자인 것으로 확인하였다. 또한, SGK1의 전사 과정이 YAP의 중요한 전사인자인 TEAD에 의하여 조절 받는다는 것을 밝혔다. 추가적으로 YAP이 SGK1의 distal enhancer에 붙어서 SGK1 의 전사를 유도한다는 것을 확인했다.

한편으로 나는 YAP과 TAZ의 활성을 조절할 수 있는 SGK1의 새로운 기능을 발견을 했다. SGK1은 YAP과 TAZ의 잘 알려진 타겟 유전자들인 CTGF와 CYR61을 조절하며 이때, SGK1의 kinase domain 과 PPxY motif가 관여한다는 것을 밝혔다. SGK1는 더 나아가 YAP과 TAZ를 통하여 CTGF와 CYR61를 조절한다. 마지막으로, 나는 SGK1이 TAZ 단백질을

안정화시키기 위하여 SGK1의 substrate인 GSK3β를 인산화시킨다는 것을 확인하였다. 그러므로 나는 SGK1이 YAP/TAZ의 새로운 positive regulator 라는 것을 밝혔다.

그에 반해, 나는 YAP이 paralog인 TAZ를 억제한다는 것을 발견했다. 우리는 이 조절이 단일방향이며 malignant transformation 여부와 상관없이 여러 종류의 세포주에서 보존된 조절이라는 것을 확인하였다. 또한 이러한 YAP에 의한 TAZ 억제는 central dogma의 번역영역에서 조절된다는 것을 밝혔다. 그리고 나는 이 조절 기작이 Hippo 신호조절체계와는 무관하다는 것을 확인했다.

결론적으로 나는 이 연구를 통해 TAZ를 활성화시키고 또 억제시키는 두 종류의 조절 기작을 발견하였다.

중심어: SGK1; YAP; TAZ; Hippo 신호전달체계
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